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<p>(21) International Application Number: PCT/GB96/01883 (22) International Filing Date: 2 August 1996 (02.08.96) (30) Priority Data: 9515941.4 3 August 1995 (03.08.95) GB (71) Applicant (for all designated States except US): ZENECA LIMITED [GB/GB]; 15 Stanhope Gate, London W1Y 6LN (GB). (72) Inventor; and (75) Inventor/Applicant (for US only): JEPSON, Ian [GB/GB]; 31 Gringer Hill, Maidenhead, Berkshire SL6 7LY (GB). (74) Agents: BISHOP, Nigel, Douglas et al.; Zeneca Agrochemicals, Intellectual Property Dept., Jealott's Hill Research Station, P.O. Box 3538, Bracknell, Berkshire RG42 6YA (GB).</p>	<p>(81) Designated States: AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).</p> <p>Published With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</p>	
<p>(54) Title: INDUCIBLE HERBICIDE RESISTANCE</p> <p>(57) Abstract</p> <p>The invention relates to DNA constructs which are capable of conferring on a plant inducible resistance to a herbicide. The inducible effect may be achieved by using a gene switch such as the <i>alcA/alcR</i> switch derived from <i>A. nidulans</i>. The invention relates in particular to inducible resistance to the herbicide N-phosphonomethyl glycine (glyphosate) and its salts.</p>		

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INDUCIBLE HERBICIDE RESISTANCE

The present invention relates to DNA constructs and plants incorporating them. In particular it relates to promoter sequences for the expression of genes which confer herbicide resistance on plants.

5 Recent advances in plant biotechnology have resulted in the generation of transgenic plants resistant to herbicide application. Herbicide tolerance has been achieved using a range of different transgenic strategies. One well documented example is the use the bacterial xenobiotic detoxifying gene phosphinothricin acetyl transferase (PAT) from *Streptomyces hydroscopicus*. Mutated genes of plant origin, for example the altered target site gene
10 encoding acetolactate synthase (ALS) from *Arabidopsis*, have been successfully utilised to generate transgenic plants resistant to herbicide application. The PAT and ALS genes have been expressed under the control of strong constitutive promoter.

We propose a system where genes conferring herbicide tolerance would be expressed in an inducible manner dependent upon application of a specific activating chemical. This
15 approach has a number of benefits for the farmer, including the following:

1. Inducible control of herbicide tolerance would alleviate any risk of yield penalties associated with high levels of constitutive expression of herbicide resistance genes. This may be a particular problem as early stages of growth where high levels of transgene product may directly interfere with normal development. Alternatively high
20 levels of expression of herbicide resistance genes may cause a metabolic drain for plant resources.
2. The expression of herbicide resistance genes in an inducible manner allows the herbicide in question to be used to control volunteers if the activating chemical is omitted during treatment.
- 25 3. The use of an inducible promoter to drive herbicide resistance genes will reduce the risk of resistant weed species becoming a major problem. If resistance genes were passed onto weed species from related crops, control could still be achieved with the herbicide in the absence of inducing chemical. This would particularly be relevant if the tolerance gene confirmed resistance to a total vegetative control herbicide which
30 would be used (with no inducing chemical) prior to sowing the crop and potentially after the crop has been harvested. For example, it can be envisaged that herbicide

resistance in cereals, such as wheat, might outcross into the weed wild oats or that herbicide resistance in oil seed rape or canola could be transferred to wild brassicas thus conferring herbicide resistance to these already troublesome weeds. A further example is that the inducible expression of herbicide resistance in sugar beet will
5 reduce the risk of wild sugar beet becoming a problem.

Several gene regulation systems (gene switches) are known and may be used for conferring inducible herbicide resistance on plants. Many such gene switches are described in the review by Gatz (Current Opinion in Biotechnology (1996) 7, 168-172) and include systems such as the tetracycline repressor gene switch, the Lac repressor system, copper
10 inducible systems such as that based on ACE 1, salicylic acid inducible promoters including the PR-1a system and systems based on steroid hormones such as the glucocorticoid, progesterone and oestrogen receptor systems. Modifications of the glucocorticoid receptor systems which include the GAL 4 binding domain from yeast and the VP16 activator are described by Aoyama *et al*, *The Plant Cell*, (1995) 7, 1773-1785 and it is envisaged that
15 similar systems may be based on, for example insect steroid hormones rather than on mammalian steroid hormones. Indeed, a system based on the ecdysone receptor of *Heliothis virescens* has recently been described. Benzene sulphonamide gene switching systems are also known (Hershey *et al*, *Plant Mol. Biol.*, 17, 679-690 (1991) as are systems based on the alcR protein from *Aspergillus nidulans* and glutathione S-transferase promoters.

20 Several genes which confer herbicide resistance are also known, for example, one herbicide to which resistance genes have been described and which is extremely widely used is N-phosphonomethyl-glycine (glyphosate) and its agriculturally acceptable salts including the isopropylamine, trimethylsulphonium, sodium, potassium and ammonium salts.

In a first aspect of the present invention there is provided a chemically inducible plant
25 gene expression cassette comprising an inducible promoter operatively linked to a target gene which confers resistance to a herbicide.

Any herbicide resistance gene may be used but genes which confer resistance to N-phosphonomethyl-glycine or salts or derivatives thereof are especially preferred.

Several inducible promoters may be used to confer the inducible resistance and these
30 include any of those listed above.

However, a particularly useful gene switch for use in this area is based on the *alc* R regulatory protein from *Aspergillus nidulans* which activates genes expression from the *alcA* promoter in the presence of certain alcohols and ketones. This system is described in our International Patent Publication No. WO93/21334 which is incorporated herein by reference.

- 5 The *alcA/alcR* gene activation system from the fungus *Aspergillus nidulans* is also well characterised. The ethanol utilisation pathway in *A. nidulans* is responsible for the degradation of alcohols and aldehydes. Three genes have been shown to be involved in the ethanol utilisation pathway. Genes *alcA* and *alcR* have been shown to lie close together on linkage group VII and *aldA* maps to linkage group VIII (Pateman JH *et al*, 1984, Proc. Soc. Lond, B217:243-264; Sealy-Lewis HM and Lockington RA, 1984, Curr. Genet, 8:253-259).
10 Gene *alcA* encodes ADHI in *A. nidulans* and *aldA* encodes AldDH, the second enzyme responsible for ethanol utilisation. The expression of both *alcA* and *aldA* are induced by ethanol and a number of other inducers (Creaser EH *et al*, 1984, Biochemical J, 255:449-454) via the transcription activator *alcR*. The *alcR* gene and a co-inducer are responsible for the
15 expression of *alcA* and *aldA* since a number of mutations and deletions in *alcR* result in the pleiotropic loss of ADHI and aldDH (Felenbok B *et al*, 1988, Gene, 73:385-396; Pateman *et al*, 1984; Sealy-Lewis & Lockington, 1984). The ALCR protein activates expression from *alcA* by binding to three specific sites in the *alcA* promoter (Kulmberg P *et al*, 1992, J. Biol. Chem, 267:21146-21153).
20 The *alcR* gene was cloned (Lockington RA *et al*, 1985, Gene, 33:137-149) and sequenced (Felenbok *et al*, 1988). The expression of the *alcR* gene is inducible, autoregulated and subject to glucose repression mediated by the CREA repressor (Bailey C and Arst HN, 1975, Eur. J. Biochem, 51:573-577; Lockington RA *et al*, 1987, Mol. Microbiology, 1:275-281; Dowzer CEA and Kelly JM, 1989, Curr. Genet, 15:457-459; Dowzer CEA and Kelly
25 JM, 1991, Mol. Cell. Biol, 11:5701-5709). The ALCR regulatory protein contains 6 cysteines near its N terminus co-ordinated in a zinc binuclear cluster (Kulmberg P *et al*, 1991, FEBS Letts, 280:11-16). This cluster is related to highly conserved DNA binding domains found in transcription factors of other ascomycetes. Transcription factors GAL4 and LAC9 have been shown to have binuclear complexes which have a cloverleaf type structure
30 containing two Zn(II) atoms (Pan T and Coleman JE, 1990, Biochemistry, 29:3023-3029; Halvorsen YDC *et al*, 1990, J. Biol. Chem, 265:13283-13289). The structure of ALCR is

similar to this type except for the presence of an asymmetrical loop of 16 residues between Cys-3 and Cys-4. ALCR positively activates expression of itself by binding to two specific sites in its promoter region (Kulmberg P *et al*, 1992, Molec. Cell. Biol, 12:1932-1939).

The regulation of the three genes, *alcR*, *alcA* and *aldA*, involved in the ethanol
5 utilisation pathway is at the level of transcription (Lockington *et al*, 1987; Gwynne D *et al*, 1987, Gene, 51:205-216; Pickett *et al*, 1987, Gene, 51:217-226).

There are two other alcohol dehydrogenases present in *A. nidulans*. ADHII is present in mycelia grown in non-induced media and is repressible by the presence of ethanol. ADHII is encoded by *alcB* and is also under the control of *alcR* (Sealy-Lewis & Lockington, 1984).
10 A third alcohol dehydrogenase has also been cloned by complementation with a *adh-* strain of *S. cerevisiae*. This gene *alcC*, maps to linkage group VII but is unlinked to *alcA* and *alcR*. The gene, *alcC*, encodes ADHIII and utilises ethanol extremely weakly (McKnight GL *et al*, 1985, EMBO J, 4:2094-2099). ADHIII has been shown to be involved in the survival of *A. nidulans* during periods of anaerobic stress. The expression of *alcC* is not repressed by the
15 presence of glucose, suggesting that it may not be under the control of *alcR* (Roland LJ and Stromer JN, 1986, Mol. Cell. Biol, 6:3368-3372).

In summary, *A. nidulans* expresses the enzyme alcohol dehydrogenase I (ADHI) encoded by the gene *alcA* only when it is grown in the presence of various alcohols and ketones. The induction is relayed through a regulator protein encoded by the *alcR* gene and
20 constitutively expressed. In the presence of inducer (alcohol or ketone), the regulator protein *activates* the expression of the *alcA* gene. The regulator protein also stimulates expression of itself in the presence of inducer. This means that high levels of the ADHI enzyme are produced under inducing conditions (i.e. when alcohol or ketone are present). Conversely, the *alcA* gene and its product, ADHI, are not expressed in the absence of inducer.
25 Expression of *alcA* and production of the enzyme is also repressed in the presence of glucose.

Thus the *alcA* gene promoter is an inducible promoter, activated by the *alcR* regulator protein in the presence of inducer (i.e. by the protein/alcohol or protein/ketone combination). The *alcR* and *alcA* genes (including the respective promoters) have been cloned and sequenced (Lockington RA *et al*, 1985, Gene, 33:137-149; Felenbok B *et al*, 1988, Gene,
30 73:385-396; Gwynne *et al*, 1987, Gene, 51:205-216).

Alcohol dehydrogenase (*adh*) genes have been investigated in certain plant species. In maize and other cereals they are switched on by anaerobic conditions. The promoter region of *adh* genes from maize contains a 300 bp regulatory element necessary for expression under anaerobic conditions. However, no equivalent to the *alcR* regulator protein has been found in any plant. Hence the *alcR/alcA* type of gene regulator system is not known in plants. Constitutive expression of *alcR* in plant cells does not result in the activation of endogenous *adh* activity.

According to a second aspect of the invention, there is provided a chemically-inducible plant gene expression cassette comprising a first promoter operatively linked to an *alcR* regulator sequence which encodes an *alcR* regulator protein, and an inducible promoter operatively linked to a target gene which confers herbicide resistance, the inducible promoter being activated by the regulator protein in the presence of an effective exogenous inducer whereby application of the inducer causes expression of the target gene.

The inducible promoter is preferably derived from the *alcA* gene promoter but may, alternatively be derived from *alcR*, *aldA* or other *alcR*-induced genes.

We have found that the *alcA/alcR* switch is particularly suited to drive herbicide tolerance genes for at least the following reasons.

1. The *alcA/alcR* switch has been developed to drive high levels of gene expression. In addition, the regulatory protein *alcR* is preferably driven from a strong constitutive promoter such as polyubiquitin. High levels of induced transgene expression, comparable to that from a strong constitutive promoter, such as 35 CaMV, can be achieved.
2. If a gene switch is to be used in a situation where the activating chemical is applied simultaneously with the herbicide, a rapid elevation in the levels of herbicide resistance gene is required. Figure 1 reveals a time course of marker gene expression (CAT) following application of inducing chemical. This study shows a rapid increase (2 hours) of CAT expression following foliar application of inducing chemical. The immediate early kinetics of induction are brought about by expressing the regulatory protein in constitutive manner, therefore no time lag is encountered while synthesis of transcription factors takes place. In addition we have chosen a simple two component system which does not rely on a complex signal transduction system.

3. We have tested the specificity of *alcA/alcR* system with a range of solvents used in agronomic practice. A hydroponic seedling system revealed that ethanol, butan-2-ol and cyclohexanone all gave high levels of induced reporter gene expression (Figure 2). In contrast when the alcohols and ketones listed in Table 1 in which are used in agronomic practice were applied as a foliar spray only ethanol gave high levels of induced reporter gene activity (Figure 3).

Table 1

1. Isobutyl methyl ketone	13. acetonyl acetone
2. Fenchone	14. JF5969 (cyclohexanone)
3. 2-heptanone	15. N-methyl pyrrolidone
4. Di-isobutyl ketone	16. polyethylene glycol
5. 5-methyl-2-hexanone	17. propylene glycol
6. 5-methylpentan-2,4-diol	18. acetophenone
7. ethyl methyl ketone	19. JF4400 (methylcyclohexanone)
8. 2-pentanone	20. propan-2-ol
9. glycerol	21. butan-2-ol
10. γ -butyrolactone	22. acetone
11. diacetone alcohol	23. ethanol
12. tetrahydrofurfuryl alcohol	24. dH ₂ O

This is of significance since illegitimate induction of transgenes will not be encountered by chance exposure to formulation solvents. Ethanol is not a common component of agrochemical formulations and therefore with appropriate spray management can be considered as a specific inducer of the *alcA / R* gene switch in a field situation.

4. A range of biotic and abiotic stresses for example pathogen infection, heat, cold, drought, wounding, flooding have all failed to induce the *alcA / alcR* switch. In addition a range of non-solvent chemical treatments for example salicylic acid, ethylene, abscisic acid, auxin, gibberelic acid, various agrochemicals, all failed to induce the *alcA / alcR* system.

The first promoter may be constitutive or tissue-specific, developmentally-programmed or even inducible. The regulator sequence, the *alcR* gene, is obtainable from *Aspergillus nidulans*, and encodes the *alcR* regulator protein.

The inducible promoter is preferably the *alcA* gene promoter obtainable from *Aspergillus nidulans* or a "chimeric" promoter derived from the regulatory sequences of the *alcA* promoter and the core promoter region from a gene promoter which operates in plant cells (including any plant gene promoter). The *alcA* promoter or a related "chimeric" promoter is activated by the *alcR* regulator protein when an alcohol or ketone inducer is applied.

The inducible promoter may also be derived from the *aldA* gene promoter, the *alcB* gene promoter or the *alcC* gene promoter obtainable from *Aspergillus nidulans*.

The inducer may be any effective chemical (such as an alcohol or ketone). Suitable chemicals for use with an *alcA/alcR*-derived cassette include those listed by Creaser *et al* (1984, Biochem J, 225, 449-454) such as butan-2-one (ethyl methyl ketone), cyclohexanone, acetone, butan-2-ol, 3-oxobutyric acid, propan-2-ol, ethanol.

The gene expression cassette is responsive to an applied exogenous chemical inducer enabling external activation of expression of the target gene regulated by the cassette. The expression cassette is highly regulated and suitable for general use in plants.

The two parts of the expression cassette may be on the same construct or on separate constructs. The first part comprises the regulator cDNA or gene sequence subcloned into an expression vector with a plant-operative promoter driving its expression. The second part comprises at least part of an inducible promoter which controls expression of a downstream target gene. In the presence of a suitable inducer, the regulator protein produced by the first part of the cassette will activate the expression of the target gene by stimulating the inducible promoter in the second part of the cassette.

In practice the construct or constructs comprising the expression cassette of the invention will be inserted into a plant by transformation. Expression of target genes in the construct, being under control of the chemically switchable promoter of the invention, may then be activated by the application of a chemical inducer to the plant.

Any transformation method suitable for the target plant or plant cells may be employed, including infection by *Agrobacterium tumefaciens* containing recombinant Ti

plasmids, electroporation, microinjection of cells and protoplasts, microprojectile transformation and pollen tube transformation. The transformed cells may then in suitable cases be regenerated into whole plants in which the new nuclear material is stably incorporated into the genome. Both transformed monocot and dicot plants may be obtained
5 in this way.

Examples of genetically modified plants which may be produced include field crops, cereals, fruit and vegetables such as: canola, sunflower, tobacco, sugarbeet, cotton, soya, maize, wheat, barley, rice, sorghum, tomatoes, mangoes, peaches, apples, pears, strawberries, bananas, melons, potatoes, carrot, lettuce, cabbage, onion.

10 The invention further provides a plant cell containing a gene expression cassette according to the invention. The gene expression cassette may be stably incorporated in the plant's genome by transformation. The invention also provides a plant tissue or a plant comprising such cells, and plants or seeds derived therefrom.

The invention further provides a method for controlling plant gene expression
15 comprising transforming a plant cell with a chemically-inducible plant gene expression cassette which has a first promoter operatively linked to an *alcR* regulator sequence which encodes an *alcA* regulator protein, and an inducible promoter operatively linked to a target gene which confers herbicide resistance, the inducible promoter being activated by the regulator protein in the presence of an effective exogenous inducer whereby application of the
20 inducer causes expression of the target gene.

This strategy of inducible expression of herbicide resistance can be achieved with a pre-spray of chemical activator or in the case of slow acting herbicides, for example N-phosphonomethyl-glycine (commonly known as glyphosate), the chemical inducer can be added as a tank mix simultaneously with the herbicide.

25 This strategy can be adopted for any resistance conferring gene/corresponding herbicide combination. For example, the *alcA/alcR* gene switch can be used with:

1. Maize glutathione S-transferase (GST-27) gene (see our International Patent Publication No WO90/08826), which confers resistance to chloroacetanilide herbicides such as acetochlor, metolachlor and alachlor.
- 30 2. Phosphinotricin acetyl transferase (PAT), which confers resistance to the herbicide commonly known as glufosinate.

3. Acetolactate synthase gene mutants from maize (see our International Patent Publication No WO90/14000) and other genes, which confer resistance to sulphonyl urea and imadazlonones.
4. Genes which confer resistance to glyphosate. Such genes include the glyphosate oxidoreductase gene (GOX) (see International Patent Publication No. WO92/00377 in the name of Monsanto Company); genes which encode for 5-enolpyruvyl-3-phosphoshikimic acid synthase (EPSPS), including Class I and Class II EPSPS, genes which encode for mutant EPSPS, and genes which encode for EPSPS fusion peptides such as that comprised of a chloroplast transit peptide and EPSPS (see for example EP 218 571, EP 293 358, WO91/04323, WO92/04449 and WO92/06201 in the name of Monsanto Company); and genes which are involved in the expression of CPLyase, Various further preferred features and embodiments of the present invention will now be described in the non-limiting examples set out below and with reference to the drawings in which:

15 Figure 1 illustrates the time course of marker gene expression (CAT) following application of inducing chemical;

Figure 2 illustrates the levels of induced reporter gene expression on root drenching with a range of solvents;

Figure 3 illustrates the levels of induced reporter gene activity when the chemicals listed in Table 1 were applied as a foliar spray;

Figure 4 illustrates the production of the 35S regulator construct by ligation of *alcR* cDNA into pJR1.

Figure 5 illustrates the production of the reporter construct;

Figure 6 is a summary of the cassettes and specific plant transformation constructs;

25 Figure 7 illustrates the chloroplast transit sequence 1 from Arabidopsis RUBISCO (CPT 1);

Figure 8 shows the sequence of plasmid pMJB1;

Figure 9 is a map of plasmid pJR1i;

Figure 10 illustrates the chloroplast transit sequence CTP2 from EPSPS class I gene from *Petunia hybrida*;

Figure 11 is a map of plasmid pUB-1;

Figure 12 is a map of plasmid pMF6;

Figure 13 is a map of plasmid pIE109 in which the numbers are in base pairs (not to scale) and the following abbreviations are used:

ADH _i	Alcohol dehydrogenase from maize;
PAT	Phosphinothricin acetyl transferase (Basta resistance gene);
AMP	Ampicillin resistance gene;
CaMV 35S	Cauliflower mosaic virus 35S promoter;
nos Poly A	Nopaline synthase poly A region;
ori	ColE1 origin of replication from pUC

Figure 14 is a map of plasmid pMV1 in which the numbers are in base pairs (not drawn to scale) and the abbreviations are as for Figure 13 with the following additional abbreviations:

UBQ _p	Maize ubiquitin promoter;
UBQ _i	Maize ubiquitin intron;
nos	Nopaline synthase 3' terminator;
CZP1 GOX	Chloroplast transit peptide - glyphosate oxidase sequence;
CZP2 GPSPS	Chloroplast transit peptide - EPSP synthetase sequence;

Figure 15 shows the preparation of plasmid pUC4 by ligation of pAr3 and pBSSK;

Figure 16 is a map of plasmid pMV2 in which the numbers are in base pairs (not drawn to scale) and the abbreviations are as for Figure 14 with the following additional abbreviations:

<i>AlcA</i>	<i>Aspergillus nidulans alcA</i> promoter;
<i>AlcR</i>	<i>Aspergillus nidulans alcR</i> promoter;

Figure 17 is a map of plasmid pDV1-pUC;

Figure 18 is a map of plasmid pDV2-pUC;

Figure 19 is a map of plasmid pDV3-Bin;

Figure 20 is a map of plasmid pDV4-Bin; and

Figure 21 is a western Blot showing the expression of EPSPS and GOX in transformants.

EXAMPLES

We have chosen to exemplify the *alcA/alcR* gene switch with genes conferring resistance to glyphosate. The switch will be used to drive inducible expression of glyphosate oxidase (GOX) in plants. Switchable GOX has been expressed alone or in conjunction with constitutive expression of 5-enol-pyruvylshikimate 3-phosphate (EPSPS) CP4. Constructs have been optimised for expression in monocotyledonous and dicotyledonous crop species.

EXAMPLE 1

Production Of The *alcR* Regulator Construct.

The *alcR* genomic DNA sequence has been published, enabling isolation of a sample of *alcR* cDNA.

The *alcR* cDNA was cloned into the expression vectors pJR1(pUC). pJR1 contains the Cauliflower Mosaic Virus 35S promoter. This promoter is a constitutive plant promoter and will continually express the regulator protein. The *nos* polyadenylation signal is in the expression vector.

Figure 4 illustrates the production of the 35S regulator construct by ligation of *alcR* cDNA into pJR1. Partial restriction of the *alcR* cDNA clone with *Bam*HI was followed by electrophoresis in an agarose gel and the excision and purification of a 2.6 Kb fragment. The fragment was then ligated into the pJR1 vector which had been restricted with *Bam*HI and phosphatased to prevent recircularisation. The *alcR* gene was thus placed under control of the CaMV 35S promoter and the *nos* 3' polyadenylation signal in this "35S-*alcR*" construct.

EXAMPLE 2

Production Of The *alcA*-CAT Reporter Construct Containing The Chimeric Promoter.

The plasmid pCaMVCN contains the bacterial chloramphenicol transferase (CAT) reporter gene between the 35S promoter and the *nos* transcription terminator (the "35S-CAT" construct).

The *alcA* promoter was subcloned into the vector pCaMVCN to produce an "*alcA*-CAT" construct. Fusion of part of the *alcA* promoter and part of the 35S promoter created a chimeric promoter which allows expression of genes under its control.

Figure 5 illustrates the production of the reporter construct. The *alcA* promoter and the 35S promoter have identical TATA boxes which were used to link the two promoters together using a recombinant PCR technique: a 246 bp region from the *alcA* promoter and

the 5' end of the CAT gene from pCaMVCN (containing part of the -70 core region of the 35S promoter) were separately amplified and then spliced together using PCR. The recombinant fragment was then restriction digested with *Bam*HI and *Hind*III. The pCaMVCN vector was partially digested with *Bam*HI and *Hind*III, then electrophoresed so
5 that the correct fragment could be isolated and ligated to the recombinant fragment.

The ligation mixtures were transformed into *E coli* and plated onto rich agar media. Plasmid DNA was isolated by miniprep from the resultant colonies and recombinant clones were recovered by size electrophoresis and restriction mapping. The ligation junctions were sequenced to check that the correct recombinants had been recovered.

10 EXAMPLE 3

Glyphosate Resistance Constructs

A summary of the cassettes and specific plant transformation constructs is shown in Figure 6.

Dicot Vector 1

15 Vector 1 is a constitutive control plasmid containing the glyphosate oxidase gene (GOX) fused to the chloroplast transit sequence 1 from Arabidopsis RUBISCO (CPT 1) (Figure 7) driven by the enhanced 35S CaMV promoter (ES) and the TMV omega translational enhancer sequence (TMV). Vector 1 utilizes the nopoline synthase terminator (nos). The synthetic GOX gene with the addition of CTP 1 was synthesised with information
20 from patent publication WO92/00377 with addition of *Nco*I site at the translation start ATG, and a *Kpn* I at the 5' end. Internal *Sph* I sites and *Nco*I site were deleted during synthesis with no change in amino acid usage. The CTP 1 GOX synthesised sequence was isolated as a *Nco* I *Kpn* I fragment and ligated using standard molecular cloning techniques into *Nco*I *Kpn*I cut pMJB1, a plasmid based on pIBT 211 containing the CaMV 35 promoter with duplicated
25 enhancer linked to the tobacco mosaic virus translational enhancer sequence replacing the tobacco etch virus 5' non-translated leader, and terminated with the nopoline synthase poly (A) signal (nos) (Figure 8).

A cassette containing enhanced 35 CaMV TMV sequence CTP1 GOX and nos terminator (dicot vector 1 pUC Figure 17) was isolated as a *Hind*III *Eco*RI fragment and
30 ligated into *Hind* III *Eco*RI cut pJRII, a Bin 19 base plant transformation vector (Figure 9).

Dicot Vector 2

The synthetic EPSPS CP4 gene, fused to the chloroplast transit sequence CTP2 (Figure 10) from EPSPS class I gene from *Petunia hybrida*, was synthesised with data from patent WO 92/04449 with *NcoI* at the translation initiation ATG. A internal *Sph I* site was silenced in the EPSPS CP4 gene with no change of amino acid usage.

A fragment containing the synthetic CTP 2 CP4 EPSPS was isolated as a *NcoI Sac I* fragment and ligated in to pMJB1. A fragment containing the CaMV 35 promoter with a duplicated enhancer, TMV omega sequence CTP 2 transit peptide, EPSPS and nos terminator was isolated as a *EcoRI Hind III* fragment (dicot vector 2 pUC Figure 18) and cloned into pJRIi to give dicot vector 2 pUC (Figure 18).

Upon sequencing the junctions of dicot vector 2, an additional sequence was identified inserted between the *SacI* site and the beginning of the nos terminator. This was as follows:

5' AGG CTG CTT GAT GAG CTC GGT ACC CGG GGA TCC ATG GAG CCG AAT 3'

Dicot Vector 3

A control vector with both EPSPS and GOX genes was constructed by cutting dicot vector 2 with *EcoRI* and inserting an Δ *EcoRI Sph I* Δ *EcoRI* linker. The sequence of the linker is shown below:

5' AAT TAG GGG CAT GCC CCT 3'

The resultant vector was cut with *Sph I* to liberate the cassette B which was cloned into an *SphI* site in dicot vector 1), 5' to the 35 CaMV promoter. Cassettes 1) and 2) were then excised as a *HindIII* and *EcoRI* fragment from dicot vector 3- pUC (Figure 19) and cloned in to pJRIi.

Dicot Vector 4

An inducible GOX vector was constructed by excising the CAT gene from "palcCAT" as *PstI* fragment. The vector band, containing the *alcA* promoter and nos terminator was gel purified and used in ligations with a *PstI-XhoI-KpnI-PstI* linker, the sequence of which is as follows:

5' GCC ACT CGA GCT AGG TAC CCT GCA 3'

The orientation of this was confirmed by sequence analysis. The TMV omega and CTPI GOX sequence from dicot vector 1) were isolated as a *XhoI KpnI* fragment and cloned into the *alcA* nos vector containing the *XhoI-KpnI-PstI* linker. The *alcA* TMV CTPI GOX

nos cassette was excised as a *HindIII* fragment and cloned into the plant transformation vector "p35S-*alcR*", containing the *alcR* cDNA nos terminator under the control of the 35 CaMV promoter to form dicot vector 4 (Figure 20).

Dicot Vector 5

5 Dicot vector 5 (Figure 22) containing inducible GOX and constitutive EPSPS genes was prepared using the following cloning strategy. Dicot vector 2 (pDV2 -pUC) was modified by cloning in a Δ *EcoRI-HindIII-ΔEcoRI* linker into the *EcoRI* site to allow excision of the CaMV en-CTP2-EPSPS -nos cassette as a *HindIII* fragment. This fragment was then ligated into *HindIII* cut pDV4-Bin. Recombinants containing all three cassettes ie 35S-*AlcR*,
10 CaMVen-CTP2-EPSPS-nos and *AlcA*-CTP1-GOX-nos were selected by hybridization with radiolabelled oligonucleotides. Confirmation of orientation was done by sequencing across all borders.

Monocot Vectors

Vector 1: Cassette D

15 An *EcoRI-NotI-EcoRI* linker (5'AATTCATTGCGGCCGCAAATG3') was inserted into dicot vector pDV1. The plasmid was cut with *NcoI* and the 5' overhang filled-in with DNA Polymerase I Klenow fragment. The linear vector was then cut with *NotI* and the resulting blunt/*NotI* fragment containing the CTP1 GOX and nos terminator was ligated into a *SmaI/NotI* digested pPUB1 vector (Figure 12) containing the polyubiquitin promoter,
20 polyubiquitin intron with a *KpnI-NotI-KpnI* linker (5'CATTGCGGCCGCAAATGGTAC3') insertion. A *HindIII-NotI-HindIII* linker (5'AGCTTGCAGCGGC CGCTGCA3') was inserted into the resulting construct.

Vector 1: Cassette E

25 An *EcoRI-NotI-EcoRI* linker (5'AATTCATTGCGGCCGCAAATG3') was inserted into dicot vector pDV2. The plasmid was cut with *NcoI* and the 5' overhang filled-in with DNA Polymerase I Klenow fragment. The linear vector was then cut with *NotI* and the resulting blunt/*NotI* fragment containing the CTP2 EPSPS and nos terminator was ligated into a *SmaI/NotI* digested pPUB1 vector containing the polyubiquitin promoter, polyubiquitin intron
30 with a *KpnI-NotI-KpnI* linker (5'CATTGCGGCCGCAAATGGT AC3') insertion to create plasmid 1. The PAT selectable marker cassette (35S CaMV promoter, *AdhI* intron,

phosphinothricin acetyl transferase gene (PAT), nos terminator) was excised from pIE108 (Figure 14) and cloned into the *Hind*III site on plasmid 1 to give monocot cassette E. Diagnostic restriction digestion was used to confirm that the selectable marker cassette was inserted 5' to 3' in the same orientation as the CTP2 EPSPS cassette.

5 A fragment containing the polyubiquitin promoter, polyubiquitin intron, CTP1 GOX and nos terminator was excised from cassette D with *Not*I and ligated into *Not*I cassette E to form monocot vector 1 (Figure 14). Restriction digestion was used to confirm that the two cassettes were inserted in the same orientation.

The selectable marker cassette (35 CaMV promoter, *Adh*I intron, phosphinothricin
10 acetyl transferase gene (PAT), nos) was excised from pIE108 and cloned into the *Hind* III site in 5) to give monocot cassette E.

Vector 1

A fragment containing the polyubiquitin promoter, polyubiquitin intron GOX and nos was
exised from cassette D with *Not*I and cloned into *Not*I cut cassette E, to form monocot vector
15 1.

Vector 2 Cassette F

An *Eco*RI fragment from pUC4 (Figure 15) containing the *alc*R cDNA and nos terminator
sequences was blunt end-filled with DNA Polymerase I Klenow fragment, ligated into pUB1
with the *Kpn*I-*Not*I-*Kpn*I linker insertion and orientated by restriction analysis. The PAT
20 selectable marker cassette was inserted in the *Hind*III site after excision from pIE108 and
orientated by restriction analysis to create vector 1. Plasmid 1 above containing the
polyubiquitin promoter, polyubiquitin intron, CTP2 EPSPS and nos terminator was cut with
*Hind*III and a Δ *Hind*III-*Not*I-*Hind*III linker:

5' AGCTCGCAGCGGCCGCTGCA3'

25 5' GCGTCGCCGGCGACGTTCA3'

inserted and orientated by sequencing to create vector 2.

A *Cla*I-*Nco*I-*Cla*I linker (5'CGATGCAGCCATGGCTGCAT3') was inserted into pMF6
(Figure 13) to give vector 3. An *Nco*I/*Kpn*I fragment containing CTP1 GOX was excised
from pDV1 and inserted into *Nco*I/*Kpn*I cut vector 3 to create vector 4. A *Sal*I fragment
30 containing the maize *Adh*I intron, CTP1 GOX was excised from vector 4 and ligated into *Sal*I
cut pUC2 containing the *alc*A promoter and nos terminator and orientated by sequencing to
create vector 5. A *Hind*III fragment from vector 5 containing the *alc*A promoter, maize *Adh*I

intron, CTP1 GOX and nos terminator was ligated into *Hind*III cut vector 2 and orientated by restriction digestion. A *Not*I fragment from the resulting construct containing polyubiquitin promoter, polyubiquitin intron, CTP2 EPSPS, nos terminator, *alcA* promoter, maize *AdhI* intron, CTP1 GOX and nos terminator was ligated into *Not*I cut vector 1 and orientated by restriction analysis to create monocot vector 2 (Figure 16).

EXAMPLE 4

Plant Transformation

Plasmids for dicot transformation were transferred to *Agrobacterium tumefaciens* LBA4404 using the freeze thaw method described by Holsters *et al* 1978.

Tobacco transformants were produced by the leaf disc method described by Bevan 1984. Shoots were regenerated on a medium containing 100 mg/l kanamycin. After rooting plants were transferred to the glasshouse and grown under 16h light/8h dark conditions.

Oilseed rape (*Brassica napus* cv westar) transformations were performed using the cotyledon petiole method described by Moloney *et al* 1989. Selection of transformed material was performed on kanamycin (15 mg/l). Rooted shoots were transferred directly to a soil based compost and grown to maturity under controlled glasshouse conditions (16h day 20°C day, 15°C night 60% RH).

Maize transformation was performed using the particle bombardment approach as described by Klein *et al* 1988. Selections were performed on 1 mg/l biolophos.

Sugar beet transformation was performed using the guard cell protoplast procedure see our International Patent Publication No. WO95/10178.

Results showing details of the transgenic plants obtained are shown in Tables 2 and 3 below.

Table 2 - Transformation Details For Tobacco

Vector	Species	Shoots removed	Rooted
pDV1	Tobacco	150	57
pDV2	Tobacco	150	60
pDV3	Tobacco	270	77
pDV4	Tobacco	350	135
pDV5	Tobacco	150	75

Table 3 - Transformation Details in Oil Seed Rape

Vector	Species	Shooting Calli	Rooted
pDV1	OSR	14	shoots from 14
pDV2	OSR	13	shoots from 13
5 pDV3	OSR	18	shoots from 18
pDV4	OSR	20	shoots from 20
pDV5	OSR	19	shoots from 18

EXAMPLE 510 **Transgenic Plant Analysis**Polymerase Chain Reaction (PCR)

Genomic DNA for PCR analysis of transgenic plants was prepared according to the method described by Edwards *et al* 1992. PCR was performed using conditions described by Jepson *et al* , Plant Molecular Biology Reporter, 9(2), 131-138 (1991). Primer sets were
 15 designed for each of the introduced cassettes.

The plants were analysed using the following oligonucleotide combinations:-

pDV1 TMV1 + GOX1, GOX3 + nos1
 pDV2 TMV1 + EPSPS1, EPSPS3 + nos1
 pDV3 EPSPS3 + GOX1
 20 pDV4 35S + AlcR1, AlcA2 + GOX1
 pDV5 35S + AlcR1, AlcA2 + GOX1, TMV1 + EPSPS1

Oligonucleotide sequences are given below:-

TMV1 5' CTCGAGTATTTTACAACAATTACCAAC
 25 GOX1 5' AATCAAGGTAACCTTGAATCCA
 GOX3 5' ACCACCAACGGTGTCTTGCTGTTGA
 NOS1 5' GCATTACATGTTAATTATTACATGCTT
 EPSPS1 5' GTGATACGAGTTTACCGCTAGCGAGAC
 EPSPS3 5' TACCTTGCGTGGACCAAGACTCC
 30 35S 5' GTCAACATGGTGGAGCACG
 AlcR1 5' GTGAGAGTTTATGACTGGAGGCGCATC
 AlcA2 5' GTCCGCACGGAGAGCCACAAACGA

Selection on Glyphosate**Kill Curves for Tobacco var Samsun and Brassica napus var Westar on glyphosate**

Both species were tested on a range of glyphosate concentrations by inserting, in the case of tobacco a 5-6mm stem segment carrying a leaf node and in the case of oil seed rape the growing tip plus two leaves into MS medium containing glyphosate at 0, 0.0055, 0.011, 0.0275, 0.055 and 0.01 mM glyphosate isopropylamine salt. The results were scored after two weeks growth as and are given in Table 4 below.

Table 4

10	Conc	Westar	Tobacco
	0	Good stem growth, 4-5 new leaves, roots up to 5cm	As OSR
	0.005	No stem growth, 1 new leaf, roots to 1cm	No growth in any organ
	0.011	No stem growth, no new leaves, roots~0.5cm	..
15	0.0275	No stem growth, no new leaves, roots~2mm	..
	0.055	No growth in any organ, ends of stem blackened	..
	0.01	As for 0.055mM	..

Selection for glyphosate tolerant transformants was performed on glyphosate concentrations of 0.01 and 0.05mM.

Constitutively tolerant plants

Following from the data obtained on wild type plants, pDV1,2 and 3 PCR +ve primary transformants were screened on MS medium containing glyphosate at the levels described above. For tobacco this was done by inserting three or four stem sections per transformant into the medium and using untransformed Samsun as control. Scoring was based on the behaviour of the majority. Plants showing tolerance at the higher concentration of herbicide were grown on to maturity in the glass house, for seed collection.

Segregation Test

Seeds were sterilized in 10% bleach for 10 min. After several washes in sterile water 200 seeds were sown on 1/2 MS medium (2.3 g/l MS salt, 1.5% sucrose, 0.8% Bactoagar,

pH 5.9) containing 100 mg/l kanamycin. Seeds were grown at 26°C with 16 hours/8 hours light/dark prior to scoring.

Western Analysis

Antibody Generation

5 GOX and EPSPS protein were over expressed in E.Coli using a pET expression system. Following IPTG induction GOX and EPSPS were electro eluted from the shake flask grown cell paste and used to immunise rabbits (two animals per clone).

Preparation of Tissue Extracts for Immunoblotting

120 mg of leaf tissue plus 60 mg PVPP and 500 µl extraction buffer (50 mM Tris-HCl pH
10 8, 1 mM EDTA, 0.3 mM DTT) were ground with a blender for several minutes. After homogenisation the extract was centrifuged at 15,000 rpm for 15 min. The supernatant was stored at -80° C until required. Protein concentrations in the extract were measured according to Bradford.

SDS-PAGE and Immunoblotting

15 25 µg protein were separated by SDS-PAGE. The running buffer was 14.4 % (w/v) glycine, 1 % (w/v) SDS and 3 % (w/v) Tris Base. The samples were loaded according to Laemmli.

After SDS-PAGE proteins were electroblotted overnight with 40 mA onto nitrocellulose (Hybond™ C, Amersham) using an electroblot unit from Biorad. The membrane was stained in 0.05 % CPTS dissolved in 12 mM HCl. Blots were rinsed in 12 mM HCl and destained for 5 - 10
20 min in 0.5 M NaHCO₃ followed by an intensive rinse with H₂O. Membranes were then blocked, immunodetected and washed according to the Amersham ECL kit. Indirect immunodetections were performed with a 1:10000 dilution of a rabbit anti-GOX or anti-EPSPS polyclonal as first antibody and with a 1:1000 dilution of an anti-rabbit second antibody, associated with horseradish peroxidase. An additional wash was carried out overnight to eliminate background. Detection was
25 performed using the ECL kit from Amersham and the results are shown in Figure 21 in which Lane (1) is the control and the remaining lanes are transformants. The western analysis demonstrates that some transformants are capable of expressing GOX and EPSPS.

Constitutively tolerant plants

Cell extracts were prepared from each glyphosate tolerant plant and the amount of
30 expressed protein estimated by western analysis using antibody appropriate to the

transformant. Plants expressing very high levels of GOX or EPSPS were tested on higher levels of glyphosate to relate level of expression to herbicide tolerance.

Inducibly tolerant plants

To demonstrate inducible tolerance to glyphosate PCR positive primary transformants from the transformations with pDV4 and 5 were transferred directly to the glass house. After two weeks the plants were induced by an ethanol root drench (5% solution) and left for 24 hours prior to western analysis performed to assess level of expression of GOX after induction. After a period of time to allow the plants to return to the uninduced state, the western analysis was repeated to allow selection of inducibly tolerant plants. Plants which showed the highest levels of GOX expression following ethanol treatment were taken forward to time course analysis. GOX levels were assessed at 6, 12, 18, 24, 36, 48 hours following ethanol treatment, by western analysis.

High expressing GOX plants for both pDV4 and pDV5 were used in glass house trials to demonstrate inducible glyphosate tolerance. Plants were induced using a range of ethanol concentrations (1-15%) by root drench application to pot grown plants. Following GOX induction plants were sprayed with glyphosate. Wild type controls and uninduced plants were also treated with herbicide.

Northern Analysis

Primary transformants containing dicots vector 2), 3), and 5). were analysed by northern blot analysis - using a CTP2 EPSPS probe as a *NcoI Sac I* fragment. Primary transformants containing the dicot vectors 1). 3). were analysed by northern blotting using a CTPI GOX probe as a *NcoI KpnI* fragment. Similarly, transgenic corn lines containing monocot vectors 1). and 2). were analysed using a CTP2 EPSPS probe.

Transformants containing dicot vector 5). or monocot vector 2). were treated with a foliar application of 5% ethanol to induce GOX levels. RNA was isolated 24 hours after treatment and subjected to northern analysis with a CTPI GOX probe.

Primary transformants which were PCR positive for the appropriate cassettes and showed GOX or EPSPS transcript levels were taken for further analysis.

Glyphosate Oxidoreductase Assay

Assays for glyphosate oxidoreductase were carried out as described by Kishore and Barry (WO 92/00377). These entailed measuring glyphosate - dependent uptake of oxygen

using an oxygen electrode, detection of glyoxylate formation by reaction with 2, 4 - dinitrophenylhydrazine and determination of the hydrozone using HPLC or, preferably, using [3- ^{14}C] - glyphosate as the substrate and detecting the formation of radioactive aminomethyl phosphonic acid via HPLC on an anion exchange column.

5 EPSPS Assay

Assays for 5-enol-pyruvylshikimate-3-phosphate (EPSP) synthase activity in plant extracts were carried out (1) by following the disappearance of the phosphoenol pyruvate substrate (as described by Rubin, J.L., Gaines, C.G and Jensen, R.A., in *Plant Physiol* (1984 75, 839-845) or (2) by conducting the assay in the reverse direction and coupling to pyruvate kinase and lactate dehydrogenase (as described by Mousdale D.M. and Coggins J.R. in *Planta* (1984) 160, 78-83) or (3) by using ^{14}C -labelled phosphoenol pyruvate as substrate and detecting the formation of radioactive EPSP by HPLC on an anion exchange column and detecting using a flow-through radioactivity detector as described by Della-Cioppa *et al* in *Proc. Nat. Acad. Sci. (USA)* (1986), 83, 6873-6877. The latter assay was used to confirm
10 that the EPSP synthase activity was, as expected, relatively resistant to inhibition by
15 glyphosate.

CLAIMS

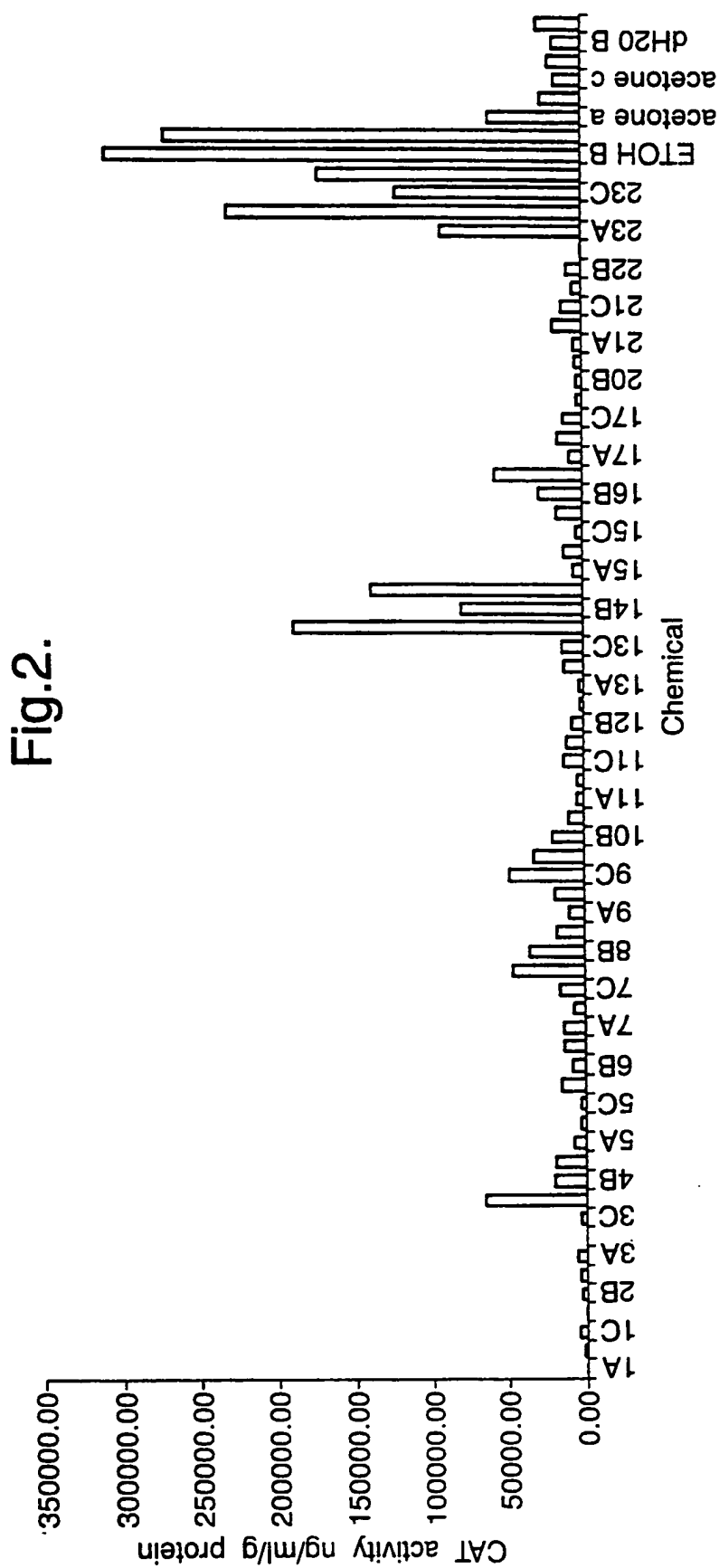
1. A chemically inducible plant gene expression cassette comprising an inducible promoter operatively linked to a target gene which confers resistance to a herbicide.
5
2. A chemically inducible plant gene expression cassette as claimed in claim 1, wherein the herbicide is N-phosphonomethyl-glycine or a salt or derivative thereof.
3. A chemically inducible plant gene expression cassette as claimed in claim 1 or claim 2,
10 wherein the inducible promoter is the tetracycline repressor gene switch, the Lac repressor system, a copper inducible systems such as that based on ACE 1, a salicylic acid inducible promoters, for example the PR-1a system, a system based on a steroid hormone such as the glucocorticoid, progesterone and oestrogen receptor systems or a modification of one of these such as a glucocorticoid receptor system which includes
15 the GAL 4 binding domain from yeast and the VP16 activator, an insect steroid hormones systems such as that based on the ecdysone receptor of *Heliothis virescens*, a benzene sulphonamide gene switching system, a gene switching based on the alcR protein from *Aspergillus nidulans* or a glutathione S-transferase promoter.
- 20 4. A chemically inducible plant gene expression cassette comprising a first promoter operatively linked to an *alcR* regulator sequence which encodes an *alcR* regulator protein, and an inducible promoter operatively linked to a target gene which confers herbicide resistance, the inducible promoter being activated by the regulator protein in the presence of an effective exogenous inducer whereby application of the inducer
25 causes expression of the target gene.
5. A plant gene expression cassette according to claim 4, wherein the inducible promoter is derived from the *alcA*, *alcR*, *aldA* or other *alcR*-induced gene promoter.
- 30 6. A plant gene expression cassette according to either of claims 4 and 5, wherein the inducible promoter is a chimeric promoter.

7. A plant gene expression cassette according to any preceding claim, wherein the target gene confers resistance to the herbicide N-phosphonomethyl-glycine or a salt or derivative thereof.
- 5
8. A plant cell containing a plant gene expression cassette according to any preceding claim.
9. A plant cell according to claim 8, wherein the plant gene expression cassette is stably incorporated in the plant's genome.
- 10
10. A plant tissue comprising a plant cell according to either of claims 8 and 9.
11. A plant comprising a plant cell according to either of claims 8 and 9.
- 15
12. A plant derived from a plant according to claim 11.
13. A seed derived from a plant according to either of claims 11 and 12.
14. A method of controlling herbicide resistance comprising transforming a plant cell with the plant gene expression cassette of any one of claims 1 to 7.
- 20
15. A method of selectively controlling weeds in a field of plants according to either of claims 11 or 12, or seeds according to claim 13, comprising applying an effective amount of the herbicide and the exogenous inducer.
- 25

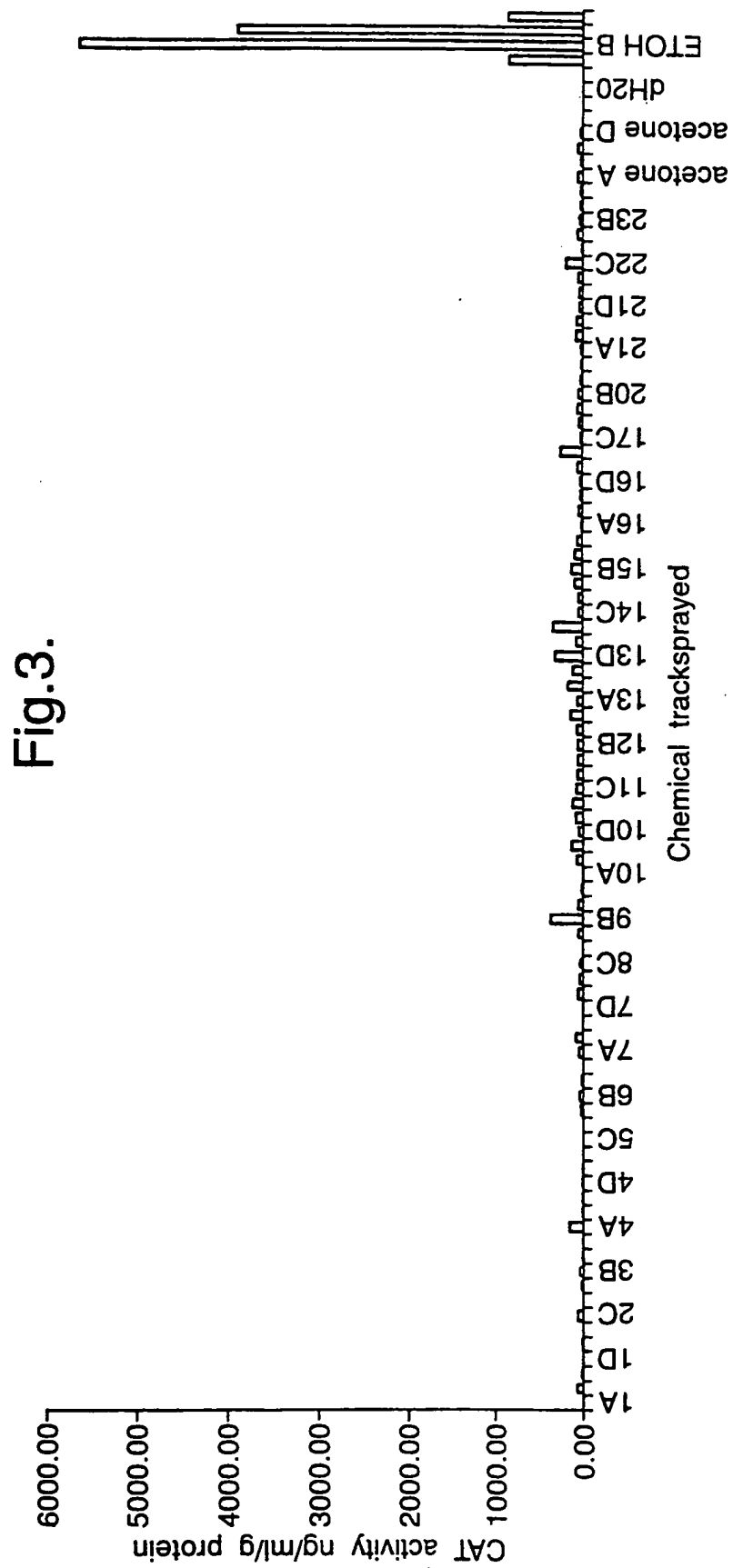
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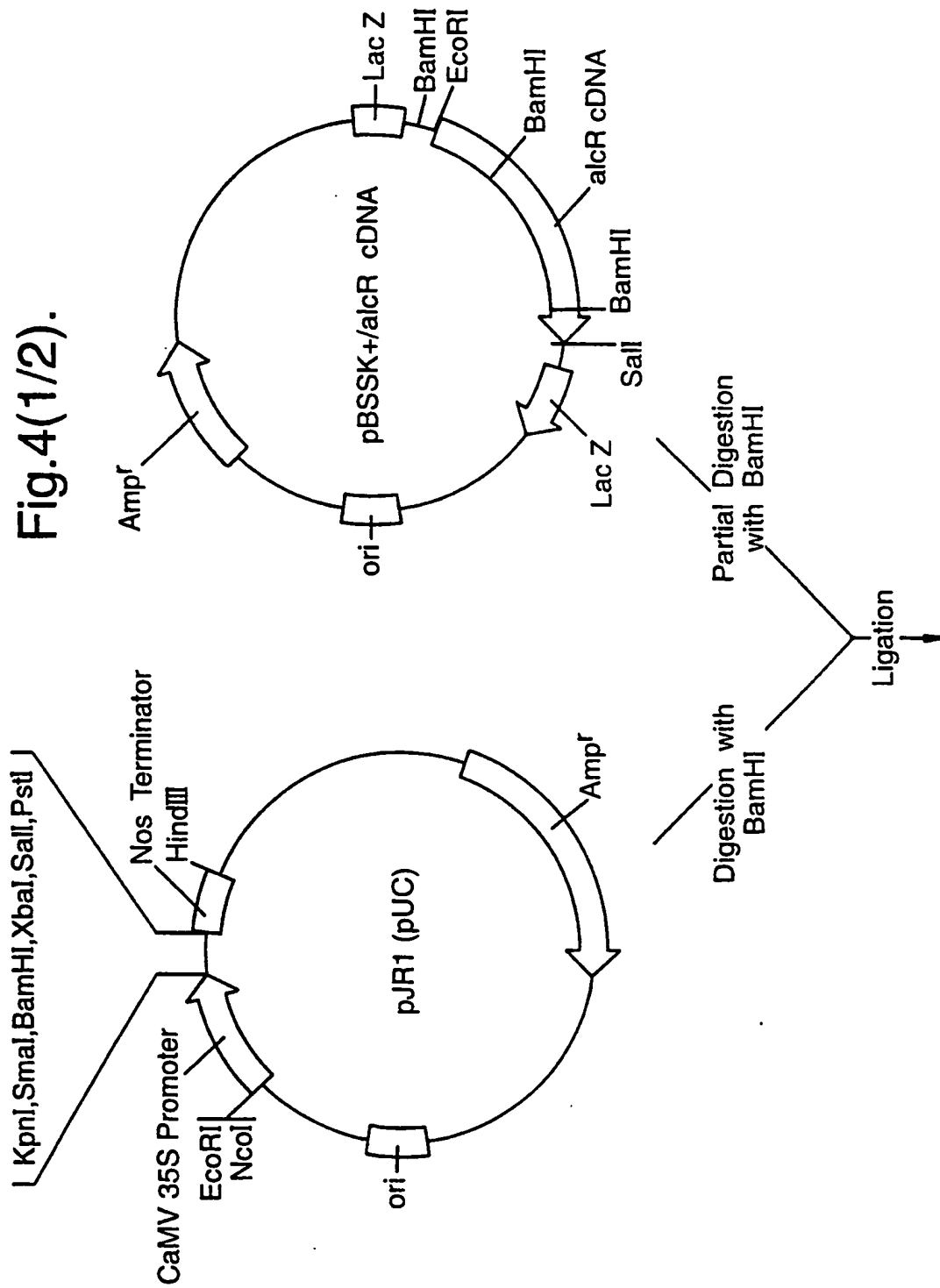


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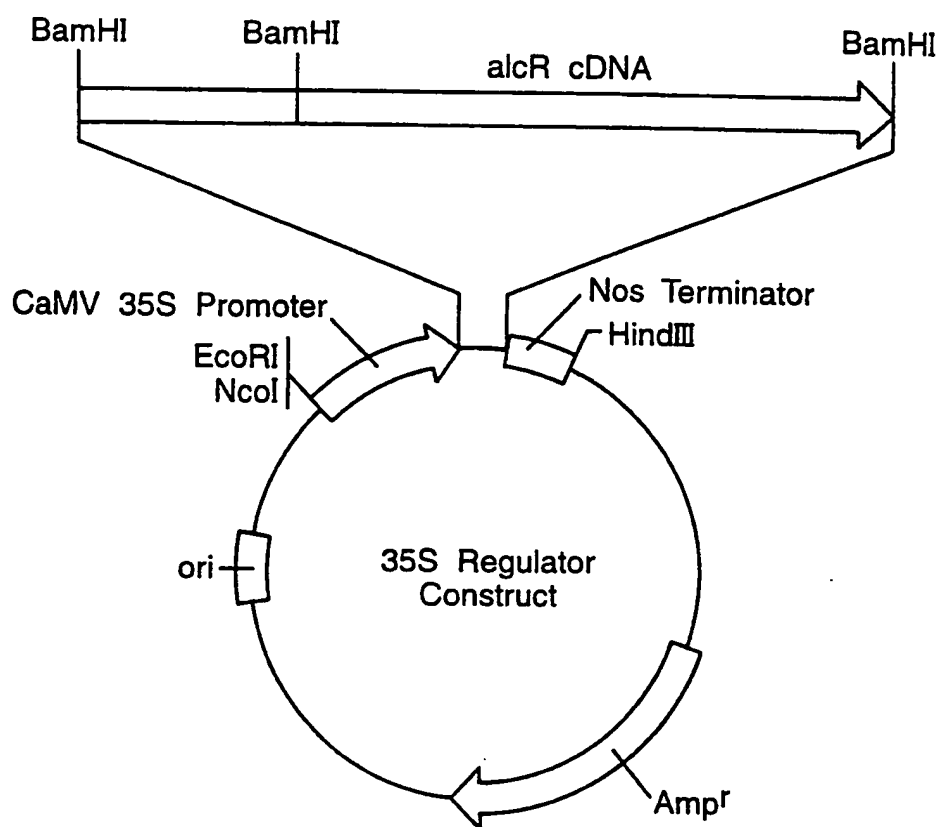
4/30

Fig.4(1/2).



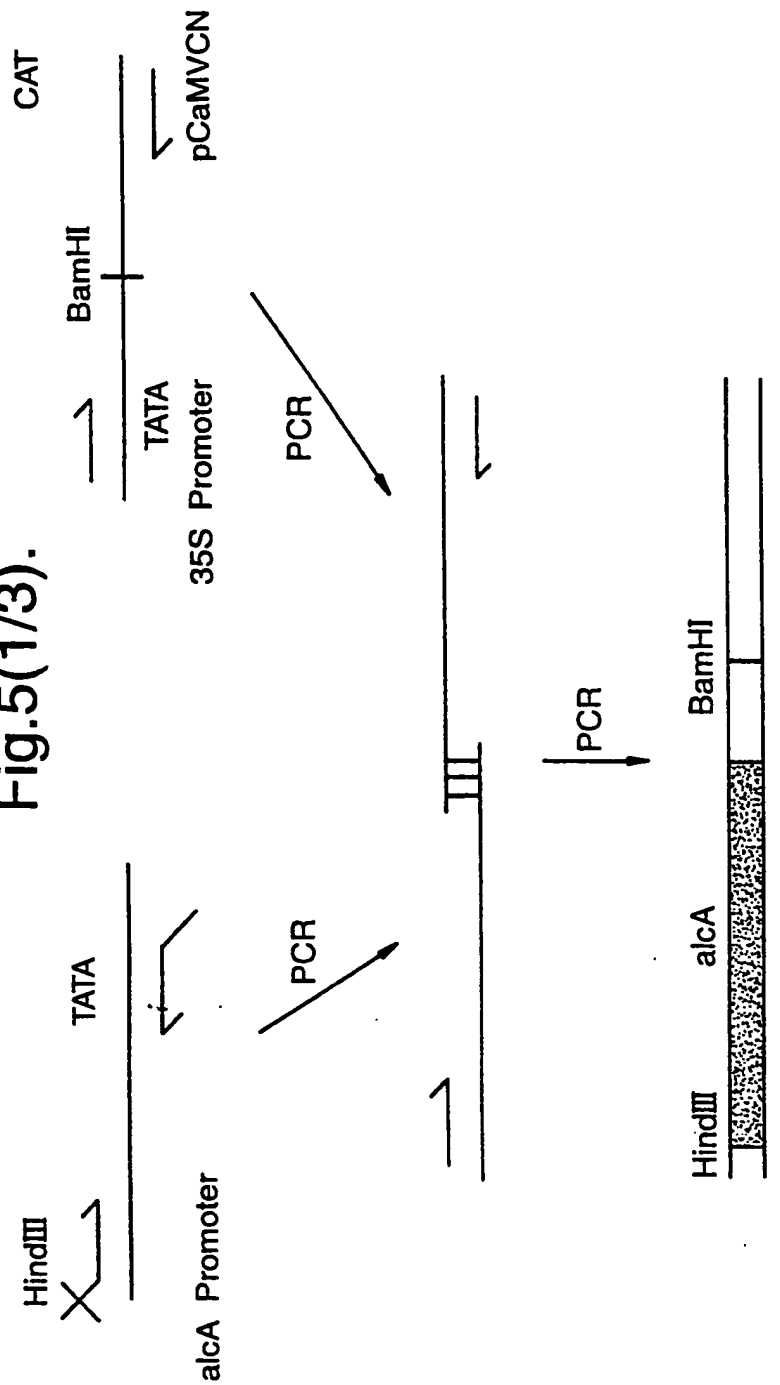
5/30

Fig.4(2/2).



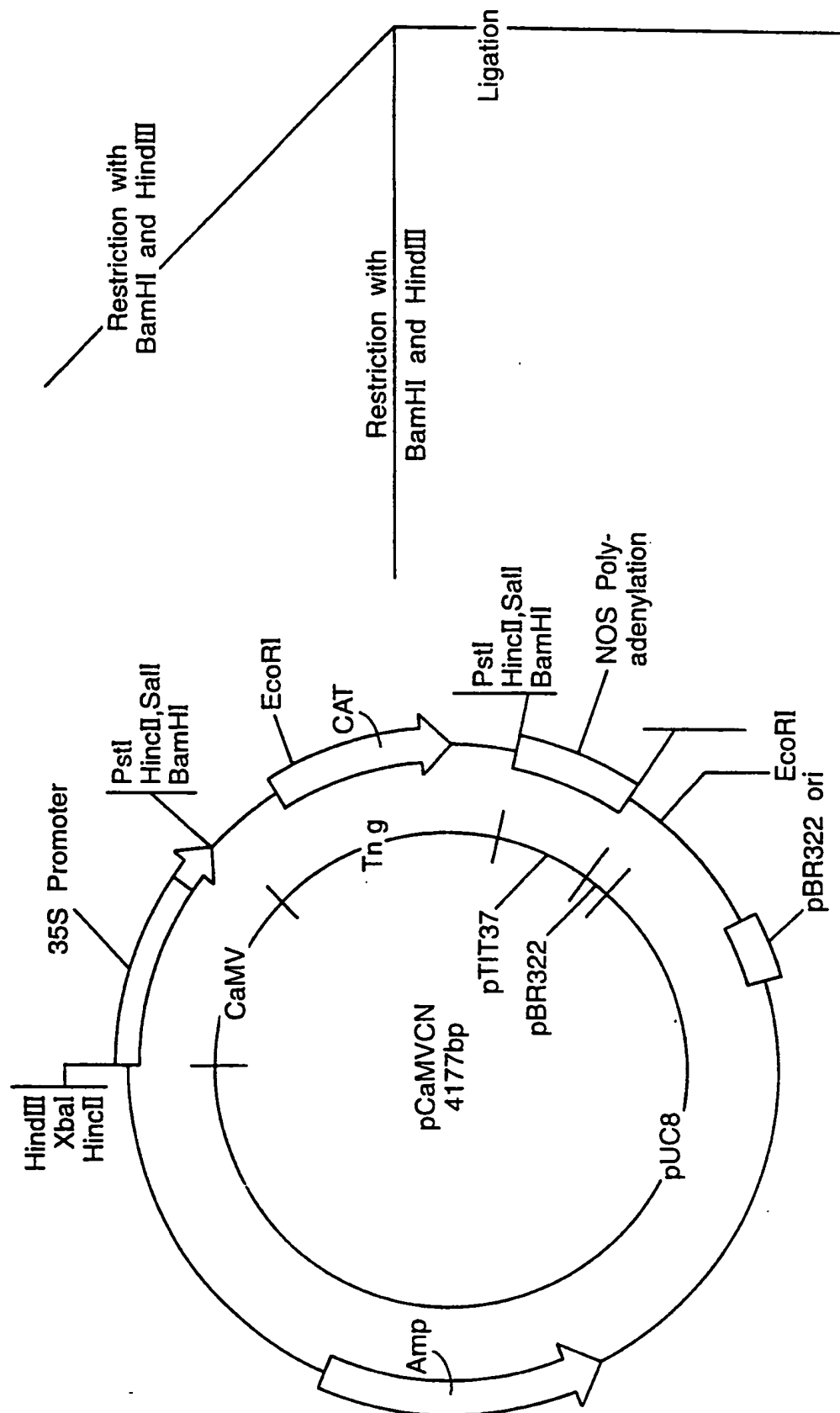
6/30

Fig.5(1/3).



7/30

Fig.5(2/3).



8/30

Fig.5(3/3).

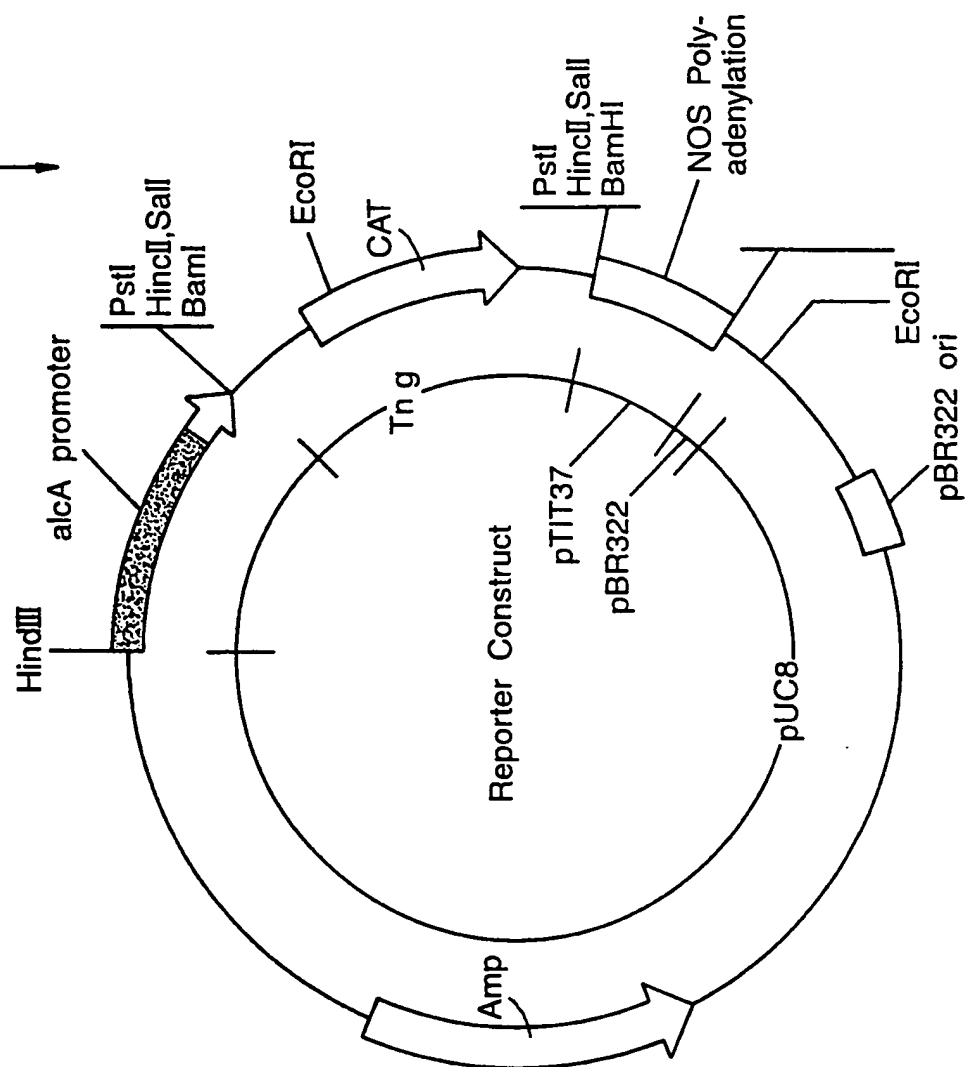
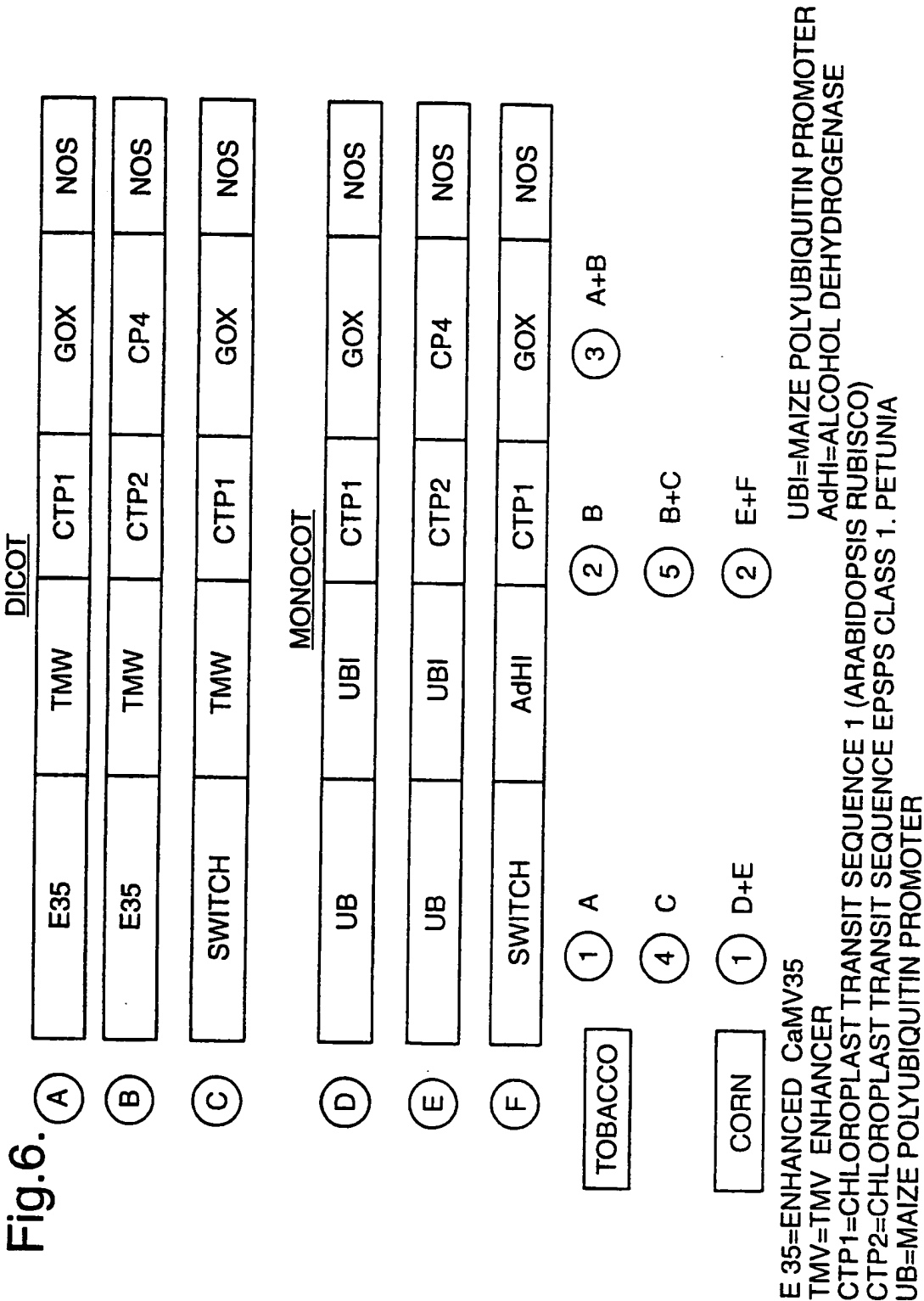


Fig.6.



10/30

Fig.7(1/3).

10	20	30	40	50	
1234567890	1234567890	1234567890	1234567890	1234567890	
AAGCTTACCA	TGGCTTCCTC	TATGCTCTCT	TCCGCTACTA	TGGTTGCCTC	50
K L T M	A S S	M L S	S A T M	V A S	
TCCGGCTCAG	GCCACTATGG	TCGCTCCTTT	CAACGGACTT	AAGTCCTCCG	100
P A Q	A T M V	A P F	N G L	K S S A	
CTGCCTTCCC	AGCCACCCGC	AAGGCTAACA	ACGACATTAC	TTCCATCACA	150
A F P	A T R	K A N N	D I T	S I T	
ACCAACGGCG	GAAGAGTTAA	CTGTATGCAG	GTGTGGCCTC	CGATTGGAAA	200
S N G G	R V N	C M Q	V W P P	I G K	
GAAGAAGTTT	GAGACTCTCT	CTTACCTTCC	TGACCTTACC	GATTCCGGTG	250
K K F	E T L S	Y L P	D L T	D S G G	
GTCGCGTCAA	CTGTATGCAG	GCTATGGCTG	AGAACCACAA	GAAGGTTGGT	300
R V N	C M Q	A M A E	N H K	K V G	
ATCGCTGGAG	CTGGAATCGT	TGGTGTTTGC	ACTGCTTTGA	TGCTTCAACG	350
I A G A	G I V	G V C	T A L M	L Q R	
TCGTGGATTG	AAGGTTACCT	TGATTGATCC	AAACCCACCA	GGTGAAGGTG	400
R G F	K V T L	I D P	N P P	G E G A	
CTTCTTTCCG	TAAAGCTGGT	TGCTTCAACG	GTTCCTCCGT	TGTTCCAATG	450
S F G	N A G	C F N G	S S V	V P M	
TCCATGCCAG	GAAACTTGAC	TAGCGTTCCA	AAGTGGCTTC	TGGATCCTGT	500
S M P G	N L T	S V P	K W L L	D P V	
TGTGAATTC					509
V N					

11/30

Fig.7(2/3).

10	20	30	40	50	
1234567890	1234567890	1234567890	1234567890	1234567890	
AAGCTTACGG	ATCCAATGGG	TCCATTGTCC	ATCCGTTTCA	GCTACTTTCC	50
K L T D	P M G	P L S	I R F S	Y F P	
AACCATCATG	CCTTGGTTGA	TTCGTTTCTT	GCTTGCTGGA	AGACCAAACA	100
T I M	P W L I	R F L	L A G	R P N K	
AGGTGAAGGA	GCAAGCTAAG	GCACTCCGTA	ACCTCATCAA	GTCCACTGTG	150
V K E	Q A K	A L R N	L I K	S T V	
CCTTTGATCA	AGTCCTTGGC	TGAGGAGGCT	GATGCTAGCC	ACCTTATCCG	200
P L I K	S L A	E E A	D A S H	L I R	
TCACGAAGGT	CACCTTACCG	TGTACCGTGG	AGAAGCAGAC	TTCGCCAAGG	250
H E G	H L T V	Y R G	E A D	F A K D	
ACCGTGGAGG	TTGGGAACTT	CGTCGTCTCA	ACGGTGTTCG	TACTCAAATC	300
R G G	W E L	R R L N	G V R	T Q I	
CTCAGCGCTG	ATGCATTGCG	TGATTTTCGAT	CCTAACTTGT	CTCACGCCTT	350
L S A D	A L R	D F D	P N L S	H A F	
TACCAAGGGA	ATCCTTATCG	AAGAGAACGG	TCACACCATC	AACCCACAAG	400
T K G	I L I E	E N G	H T I	N P Q G	
GTCTCGTGAC	TCTCTTGTTT	CGTCGTTTCA	TCGCTAACGG	TGGAGAGTTC	450
L V T	L L F	R R F I	A N G	G E F	
GTGTCGCTC	GTGTTATCGG	ATTCGAGACT	GAAGGTCGTG	CTCTCAAGGG	500
V S A R	V I G	F E T	E G R A	L K G	
TATCACCACC	ACCAACGGTG	TTCTTGCTGT	TGATGCTGCA	GTGTTGIGAA	550
I T T	T N G V	L A V	D A A	V L . I	
TTC					553

12/30

Fig.7(3/3).

10	20	30	40	50	
1234567890	1234567890	1234567890	1234567890	1234567890	
AAGCTTACTG	CAGTTGTTGC	AGCTGGTGCA	CACTCCAAGT	CTCTTGCTAA	50
K L T A	V V A	A G A	H S K S	L A N	
CTCCCTTGGT	GATGACATCC	CATTGGATAC	CGAACGTGGA	TACCACATCG	100
S L G	D D I P	L D T	E R G	Y H I V	
TGATCGCCAA	CCCAGAAGCT	GCTCCACGTA	TTCCAACACTAC	CGATGCTTCT	150
I A N	P E A	A P R I	P T T	D A S	
GGAAAGTTCA	TCGCTACTCC	TATGGAGATG	GGTCTTCGTG	TIGCTGGAAC	200
G K F I	A T P	M E M	G L R V	A G T	
CGTTGAGTTC	GCTGGTCTCA	CTGCTGCTCC	TAACTGGAAG	CGTGCTCACG	250
V E F	A G L T	A A P	N W K	R A H V	
TTCTCTACAC	TCGTGCTCGT	AAGTTGCTTC	CAGCTCTCGC	TCCTGCCAGT	300
L Y T	R A R	K L L P	A L A	P A S	
TCTGAAGAAC	GTTACTCCAA	GTGGATGGGT	TTCCGTCCAA	GCATCCCAGA	350
S E E R	Y S K	W M G	F R P S	I P D	
TTCCCTTCCA	GTGATTGGTC	GTGCTACCCG	TACTCCAGAC	GTTATCTACG	400
S L P	V I G R	A T R	T P D	V I Y A	
CTTTCGGTCA	CGGTCACCTC	GGTATGACTG	GTGCTCCAAT	GACCGCAACC	450
F G H	G H L	G M T G	A P M	T A T	
CTCGTTTCTG	AGCTCCTCGC	AGGTGAGAAG	ACCTCTATCG	ACATCTCTCC	500
L V S E	L L A	G E K	T S I D	I S P	
ATTCGCACCA	AACCGTTTCG	GTATTGGTAA	GTCCAAGCAA	ACTGGTCCTG	550
F A P	N R F G	I G K	S K Q	T G P A	
CATCCTAAGG	TACCGAATTC				570
S . G	T E F				

13/30

Fig.8.

	10	20	30	40	50	60	
	HindIII	SphI	PstI				
			→35S				
1	AAGCTTGCAT	GCCTGCAGGT	CAACATGGTG	GAGCACGACA	CACTTGTCTA	CTCCAAAAAT	
	TTCGAACGTA	CGGACGTC	CA	CTCGTGCTGT	GTGAACAGAT	GAGGTTTTTA	
61	ATCAAAGATA	CAGTCTCAGA	AGACCAAAGG	GCAATTGAGA	CTTTTCAACA	AAGGTAATA	
	TAGTTTCTAT	GTCAGAGTCT	TCTGGTTTCC	CGTTAACTCT	GAAAGTTGT	TTCCCAATTAT	
121	TCCGGAAACC	TCCTCGGATT	CCATTGCCCCA	GCTATCTGTC	ACTTTATTGT	GAAGATAGTG	
	AGGCCTTTGG	AGGAGCCTAA	GGTAACGGGT	CGATAGACAG	TGAAATAACA	CTTCTATCAC	DUF ATC EN
181	GAAAGGAAG	GTGGCTCCTA	CAAATGCCAT	CATTGCCGATA	AAGGAAAGGC	CATCGTTGAA	
	CTTTTCCTTC	CACCGAGGAT	GTTACGGTA	GTAACGCTAT	TTCCCTTCCG	GTAGCAACTT	
241	GATGCCTCTG	CCGACAGTGG	TCCCAAAGAT	GGACCCCCAC	CCACGAGGAG	CATCGTGGAA	
	CTACGGAGAC	GGCTGTCACC	AGGGTTTCTA	CCTGGGGGTG	GGTGCTCCTC	GTAGCACCTT	

14/30

Fig. 8 (Cont).

301 AAAAGAAGAC GTTCCAACCA CGTCTTCAAA GCAAGTGGAT TGATGTGATA ACATGGTGGA
 TTTTCTTCTG CAAGGTGGT GCAGAAAGTTT CGTTCACCTA ACTACACTAT TGTACCACCT
 361 GCACGACACA CTTGTCTACT CCAAAAATAT CAAAGATACA GTCTCAGAAG ACCAAAGGGC
 CGTGCTGTGT GAACAGATGA GGTTTTATA GTTCTATGT CAGAGTCTTC TGGTTTCCCG
 421 AATTGAGACT TTTCACAAA GGGTAATATC CGGAAACCTC CTCGGATTCC ATTGCCCCAGC
 TTAACCTCTGA AAAGTTGTTT CCCATTATAG GCCTTTGGAG GAGCCTAAGG TAACGGGTGCG
 481 TATCTGTCAC TTTATTGTGA AGATAGTGA AAAGGAAGGT GGCTCCTACA AATGCCATCA
 ATAGACAGTG AAATAACACT TCTATCACCT TTTCCTTCCA CCGAGGATGT TTACGGTAGT
 541 TTGCGATAAA GGAAAGGCCA TCGTTGAAGA TGCCTCTGCC GACAGTGGTC CCAAAGATGG
 AACGCTATTT CCTTCCGGT AGCAACTTCT ACGGAGACGG CTGTCACCCAG GGTTCCTACC
 601 ACCCCCACCC ACGAGGAGCA TCGTGGA AAAAAGAAGACGT TCCAACCACG TCTTCAAAGC
 TGGGGGTGGG TGCTCCTCGT AGCACCTTTT TTCTTCTGCA AGGTTGGTGC AGAAGTTTCG
 661 AAGTGGATTG ATGTGATATC TCCACTGACG TAAGGGATGA CGCACATCC CACTATCCTT
 TTCACCTAAC TACACTATAG AGGTGACTGC ATTCCCCTACT GCCTGTTAGG GTGATAGGAA

35S
PR

EcoRV
Promoter

15/30

Fig. 8 (Cont i).

721 CGCAAGACCC TTCCTCTATA TAAGGAAGTT CATTTCAATT GGAGAGGACC TCGAGTATTT
 GCGTTCTGGG AAGGAGATAT ATTCTTCAA GTAAAGTAAA CCTCTCTGG AGTCATATAA
 TATA BOX
 781 TTACAACAAT TACCAACAAC AACAAACAAC AAACAACATT ACAATTACTA TTTACAATTA
 AATGTTGTTA ATGGTTGTTG TTGTTTGTG TTTGTTGTAA TGTTAATGAT AAATGTTAAT
 NcoI SmaI KpnI SacI
 841 CACCATGGAT CCGGGGTAC CGAGCTCGAA TTTCCCCGAT CGTCAAACA TTTGGCAATA
 GTGGTACCTA GGGGCCATG GCTCGAGCTT AAAGGGGCTA GCAAGTTTGT AAACCGTTAT
 BamHI
 901 AAGTTTCTTA AGATTGAATC CTGTTGCCGG TCTTGCGATG ATTATCATAT AATTCTGT
 TTCAAAGAAT TCTAACTTAG GACAACGGCC AGAACGCTAC TAATAGTATA TTAAGACAA
 961 GAATTACGTT AAGCATGTAA TAATTAACAT GTAATGCATG ACGTTATTTA TGAGATGGGT
 CTTAATGCAA TTCGTACATT ATTAATTGTA CATTACGTAC TGCAATAAAT ACTCTACCCA
 1021 TTTTATGATT AGAGTCCCGC AATTATACAT TTAATACGG ATAGAAAACA AAATATAGCG
 AAAATACTAA TCTCAGGGCG TTAATATGTA AATTATGCG TATCTTTTGT TTTATATCGC
 1081 CGCAAACTAG GATAAATTAT CGCGCGCGGT GTCATCTATG TTAGTAGATC GGGAATTC
 GCGTTTGATC CTATTTAATA CGCGCGGCCA CAGTAGATAC AATGATCTAG CCCTTAAG
 EcoRI

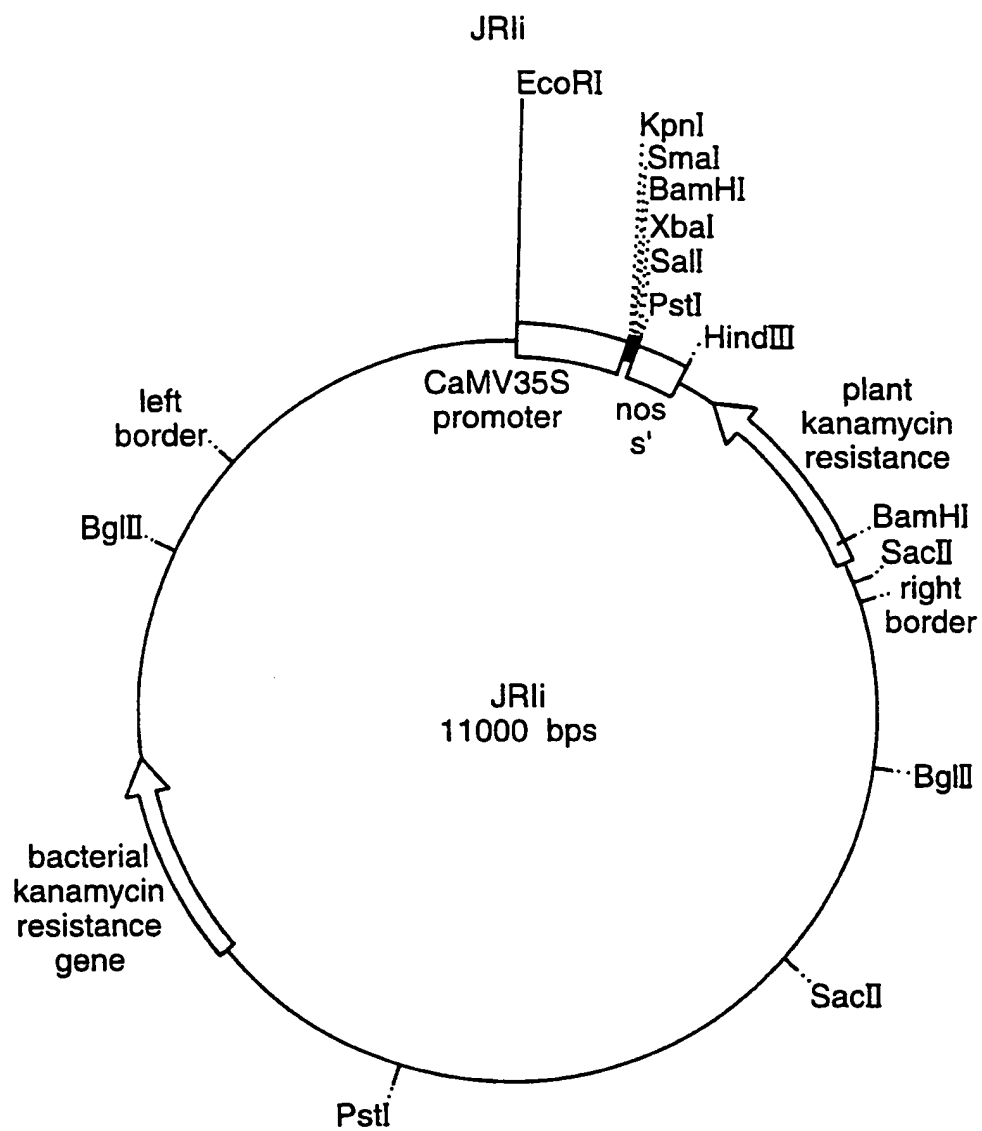
Total number of bases is: 1138.

DNA sequence composition: 370 A; 253 C; 234 G; 281 T; 0 OTHER;

Sequence name: PMJB1

16/30

Fig.9.



17/30

Fig.10(1/4).

10	20	30	40	50	
1234567890	1234567890	1234567890	1234567890	1234567890	
AAGCTTACCA	TGGCTCAAGT	TAGCAGAATC	TGCAATGGTG	TGCAGAACCC	50
K L T M	A Q V	S R I	C N G V	Q N P	
ATCTCTTATC	TCCAATCTCT	CGAAATCCAG	TCAACGCAAA	TCTCCCTTAT	100
S L I	S N L S	K S S	Q R K	S P L S	
CGGTTTCTCT	GAAGACGCAG	CAGCATCCAC	GAGCTTATCC	GATTTCGTCC	150
V S L	K T Q	Q H P R	A Y P	I S S	
TCGTGGGGAT	TGAAGAAGAG	TGGGATGACG	TTAATTGGCT	CTGAGCTTCG	200
S W G L	K K S	G M T	L I G S	E L R	
TCCCTCTTAAG	GTCATGTCTT	CTGTTTCCAC	GGCGTGTATG	CTTCACGGTG	250
P L K	V M S S	V S T	A C M	L H G A	
CAAGCAGCCG	TCCAGCAACT	GCTCGTAAGT	CCTCTGGTCT	TTCTGGAACC	300
S S R	P A T	A R K S	S G L	S G T	
GTCCGTATTTC	CAGGTGACAA	GTCTATCTCC	CACAGGTCTT	TCATGTTTGG	350
V R I P	G D K	S I S	H R S F	M F G	
AGGTCTCGCT	AGCGGTGAAA	CTCGTATCAC	CGGTCTTTTG	GAAGGTGAAG	400
G L A	S G E T	R I T	G L L	E G E D	
ATGTTATCAA	CACTGGTAAG	GCTATGCAAG	CTATGGGTGC	CAGGATCCTG	450
V I N	T G K	A M Q A	M G A	R I L	
TTGTGAATTC					460
L . I					

Fig.10(2/4). 18/30

10	20	30	40	50	
1234567890	1234567890	1234567890	1234567890	1234567890	
AAGCTTAGGA	TCCGTAAGGA	AGGTGATACT	TGGATCATTG	ATGGTGTTGG	50
K L R I	R K E	G D T	W I I D	G V G	
TAACGGTGGA	CTCCTTGCTC	CTGAGGCTCC	TCTCGATTTC	GGTAACGCTG	100
N G G	L L A P	E A P	L D F	G N A A	
CAACTGGTTG	CCGTTTGA CT	ATGGGTCTTG	TTGGTGTTTA	CGATTTCGAT	150
T G C	R L T	M G L V	G V Y	D F D	
AGCACTTTCA	TTGGTGACGC	TTCTCTCACT	AAGCGTCCAA	TGGGTGCTGT	200
S T F I	G D A	S L T	K R P M	G R V	
GTTGAACCCA	CTTCGCGAAA	TGGGTGTGCA	GGTGAAGTCT	GAAGACGGTG	250
L N P	L R E M	G V Q	V K S	E D G D	
ATCGTCTTCC	AGTTACCTTG	CGTGGACCAA	AGACTCCAAC	GCCAATCACC	300
R L P	V T L	R G P K	T P T	P I T	
TACAGGGTAC	CTATGGCTTC	CGCTCAAGTG	AAGTCCGCTG	TTCTGCTTGC	350
Y R V P	M A S	A Q V	K S A V	L L A	
TGGTCTCAAC	ACCCCAGGTA	TCACCACTGT	TATCGAGCCA	ATCATGACTC	400
G L N	T P G I	T T V	I E P	I M T R	
GTGACCACAC	TGAAAAGATG	CTTCAAGGTT	TTGGTGCTAA	CCTTACCGTT	450
D H T	E K M	L Q G F	G A N	L T V	
GAGACTGATG	CTGACGGTGT	GCGTACCATC	CGTCTTGAAG	GTCGTGGTAA	500
E T D A	D G V	R T I	R L E G	R G K	
GCTCACCGGT	CAAGTGATTG	ATGTTCCAGG	TGATCCATCC	TCTACTGCTT	550
L T G	Q V I D	V P G	D P S	S T A F	
TCCCATTGGT	TGCTGCCTTG	CTTGTTCCAG	GTTCCGACGT	CACCATCCTT	600
P L V	A A L	L V P G	S D V	T I L	
AACGTTTTGA	TGAACCCAAC	CCGTACTGGT	CTCATCTTGA	CTCTGCAGTG	650
N V L M	N P T	R T G	L I L T	L Q C	

19/30

Fig.10(3/4).

10	20	30	40	50
1234567890	1234567890	1234567890	1234567890	1234567890
TTGTGAATTC				
C	E	F		660

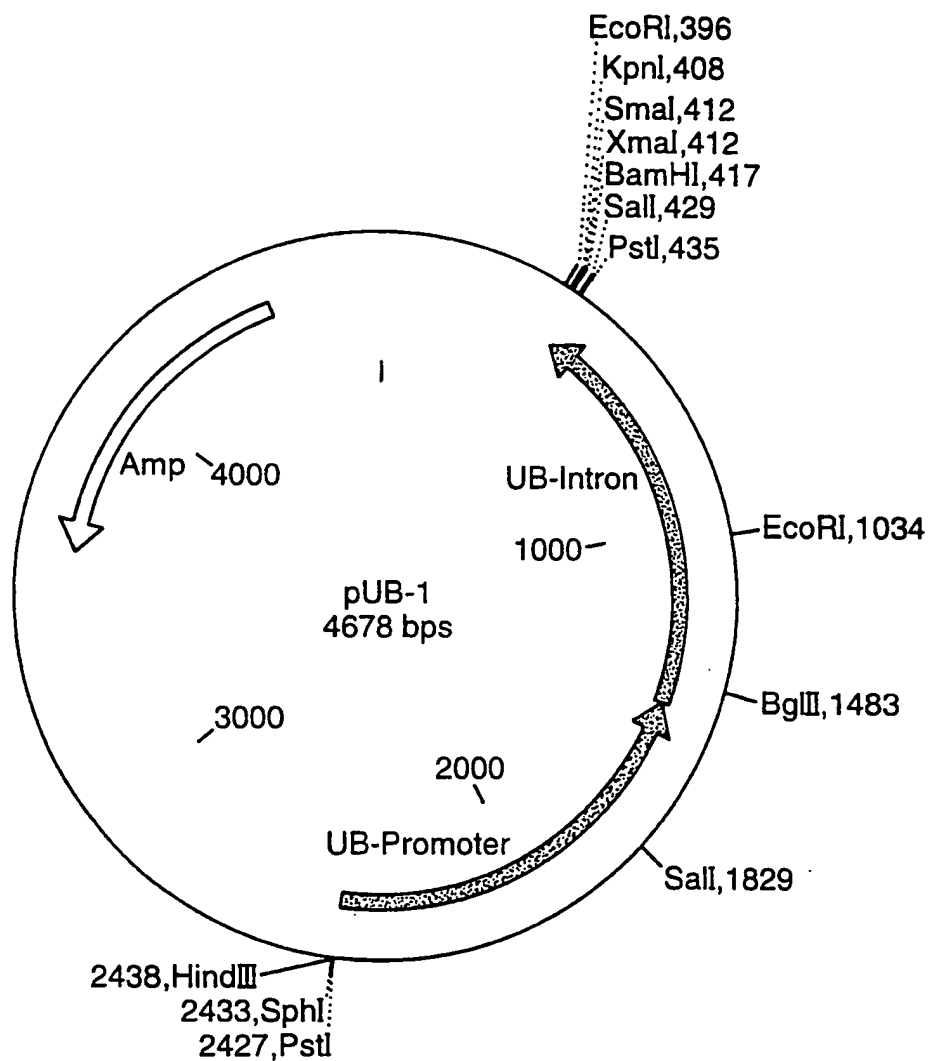
20/30

Fig.10(4/4).

10	20	30	40	50	
1234567890	1234567890	1234567890	1234567890	1234567890	
AAGCTTCTGC	AGGAAATGGG	TGCCGACATC	GAAGTGATCA	ACCCACGTCT	50
K L L Q	E M G	A D I	E V I N	P R L	
TGCTGGTGGA	GAAGACGTGG	CTGACTTGCG	TGTTGTTTCT	TCTACTTTGA	100
A G G	E D V A	D L R	V R S	S T L K	
AGGGTGTAC	TGTTCCAGAA	GACCGTGCTC	CTTCTATGAT	CGACGAGTAT	150
G V T	V P E	D R A P	S M I	D E Y	
CCAATTCTCG	CTGTTGCAGC	TGCATTGCTT	GAAGGTGCTA	CCGTTATGAA	200
P I L A	V A A	A F A	E G A T	V M N	
CGGTTTGGA	GAAGTCCGTG	TTAAGGAAAG	CGACCGTCTT	TCTGCTGTCTG	250
G L E	E L R V	K E S	D R L	S A V A	
CAAACGGTCT	CAAGCTCAAC	GGTGTGATT	GCGATGAAGG	TGAGACTTCT	300
N G L	K L N	G V D C	D E G	E T S	
CTCGTCGTGC	GTTGTCGTCC	TGACGGTAAG	GGTCTCGGTA	ACGCTTCTGG	350
L V V R	G R P	D G K	G L G N	A S G	
AGCAGCTGTC	GCTACCCACC	TCGATCACCG	TATCGCTATG	AGCTTCCTCG	400
A A V	A T H L	D H R	I A M	S F L V	
TTATGGGTCT	CGTTTCTGAA	AACCCTGTTA	CTGTTGATGA	TGCTACTATG	450
M G L	V S E	N P V T	V D D	A T M	
ATCGCTACTA	GCTTCCCAGA	GTTTCATGGAT	TTGATGGCTG	GTCTTGGAGC	500
I A T S	F P E	F M D	L M A G	L G A	
TAAGATCGAA	CTCTCCGACA	CTAAGGCTGC	TTGATGAGCT	CGAATTC	547
K I E	L S D T	K A A	. . A	R I	

21/30

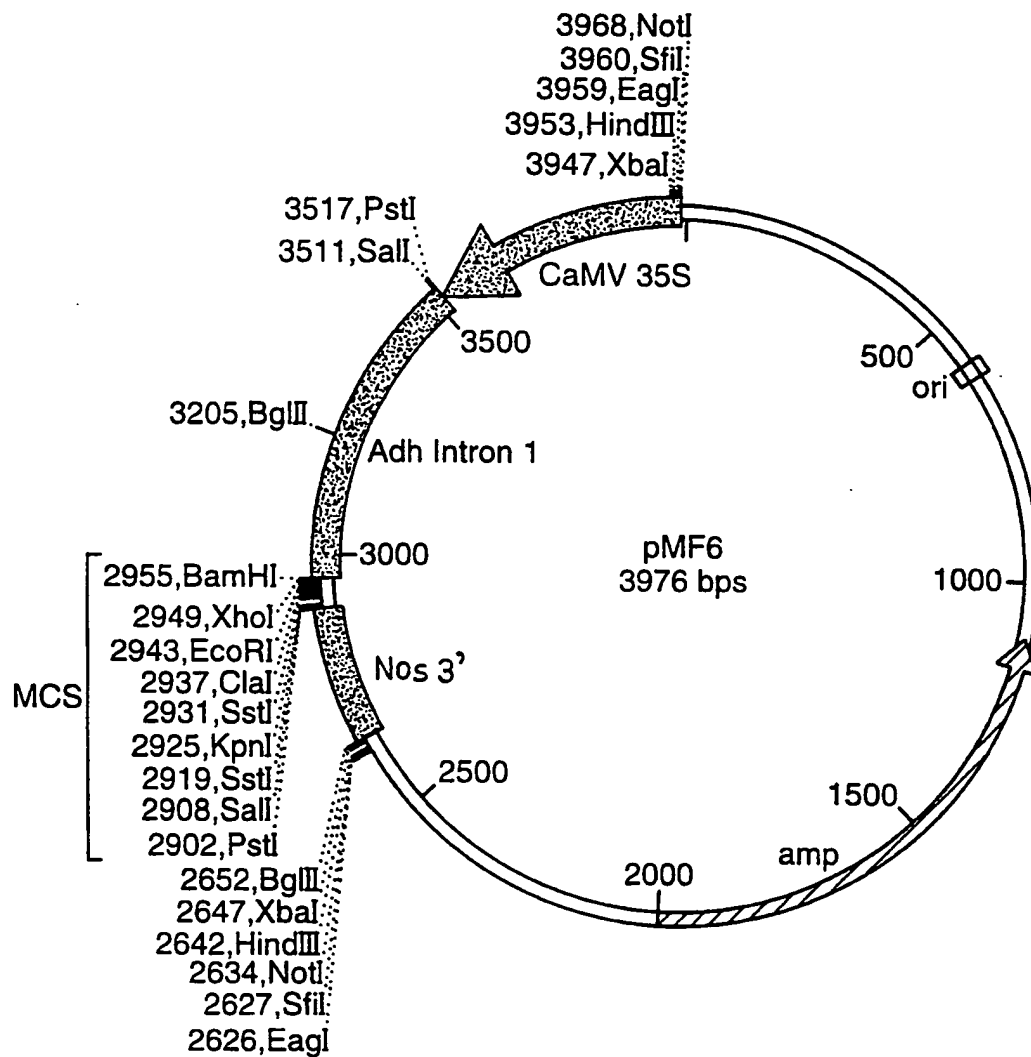
Fig.11.



Ubiquitin promoter fragment PCR'd from maize.
 2 Kb. fragment cloned into pUC19.
 Junctions have been sequenced to confirm that it
 is the Ubiquitin promoter.

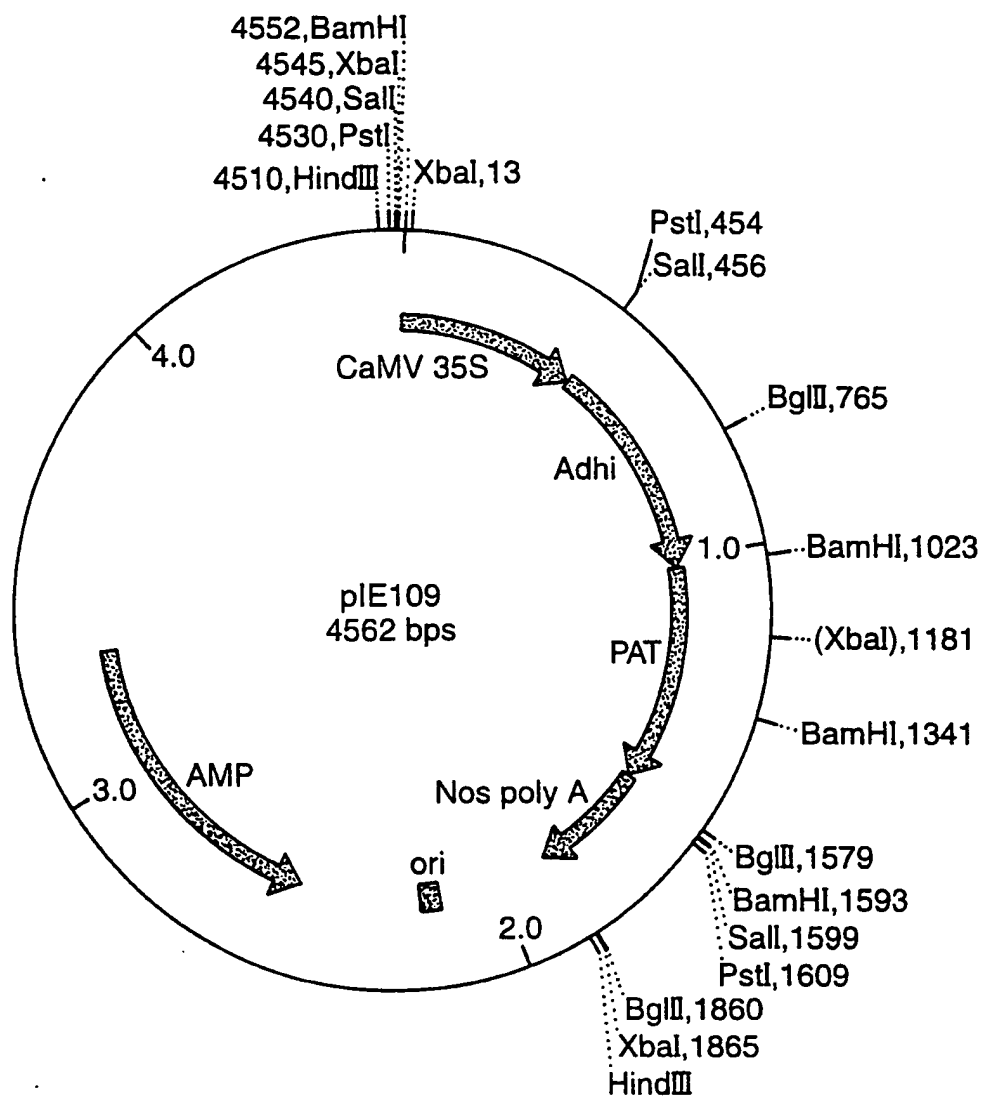
22/30

Fig.12.



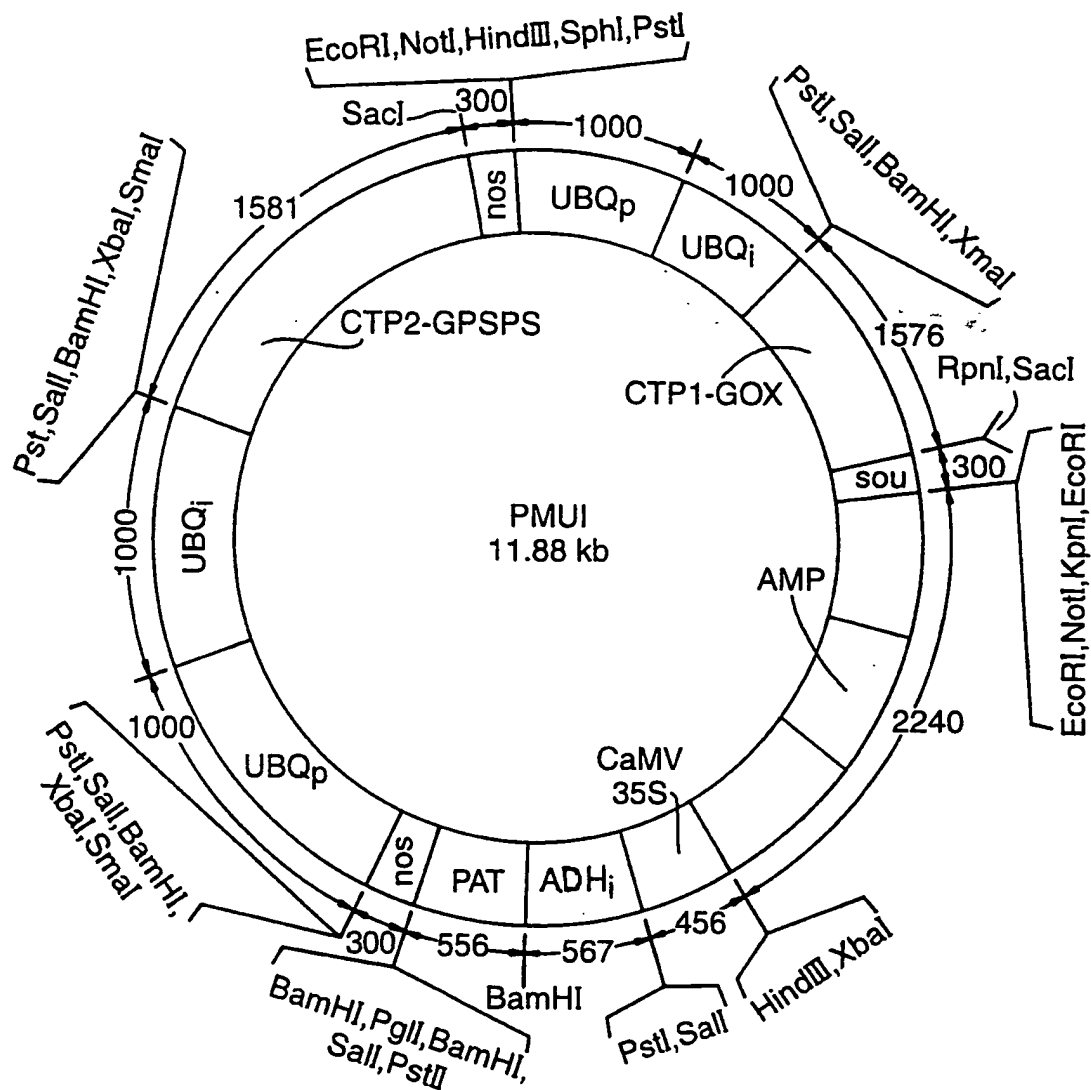
23/30

Fig.13.



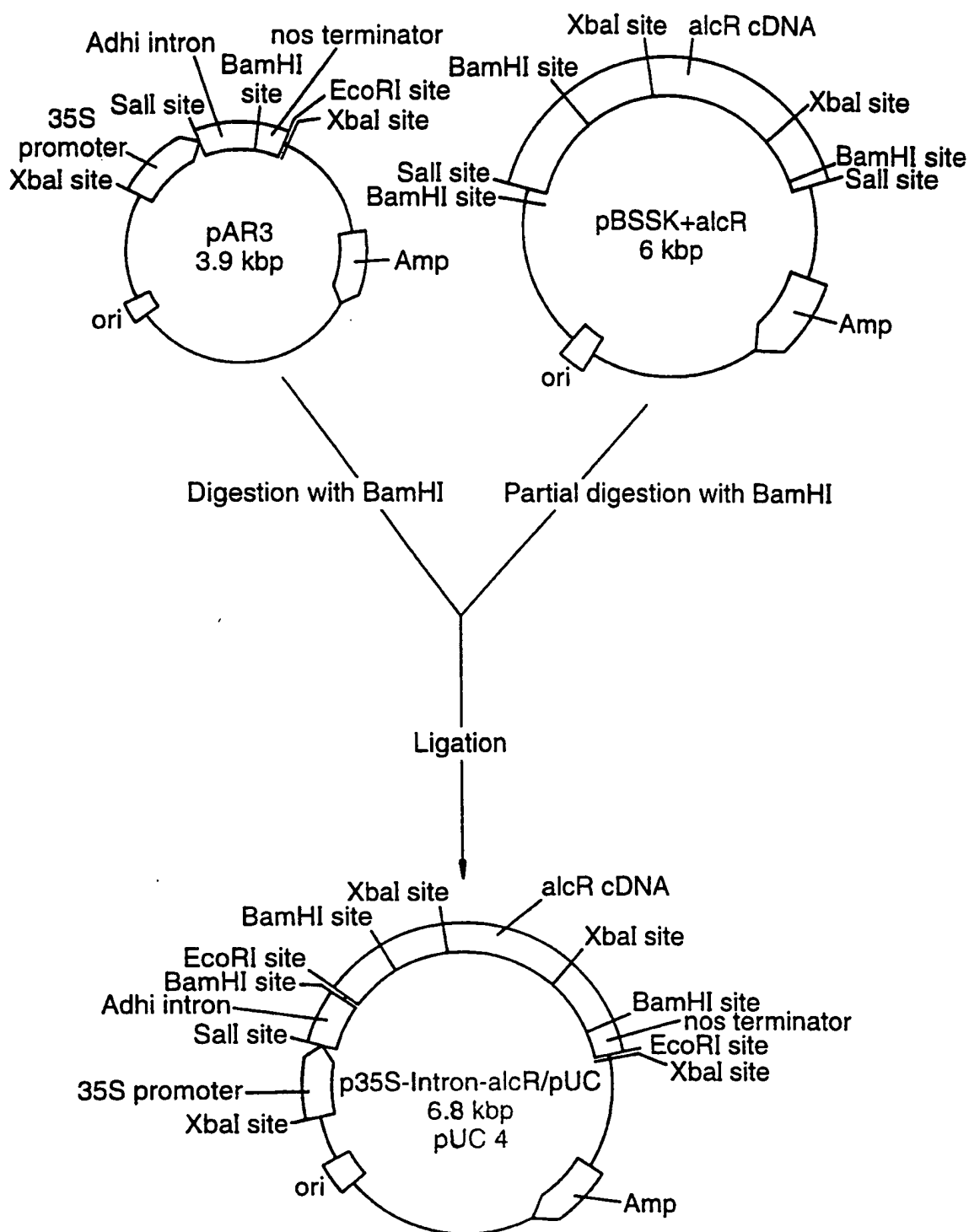
24/30

Fig.14.



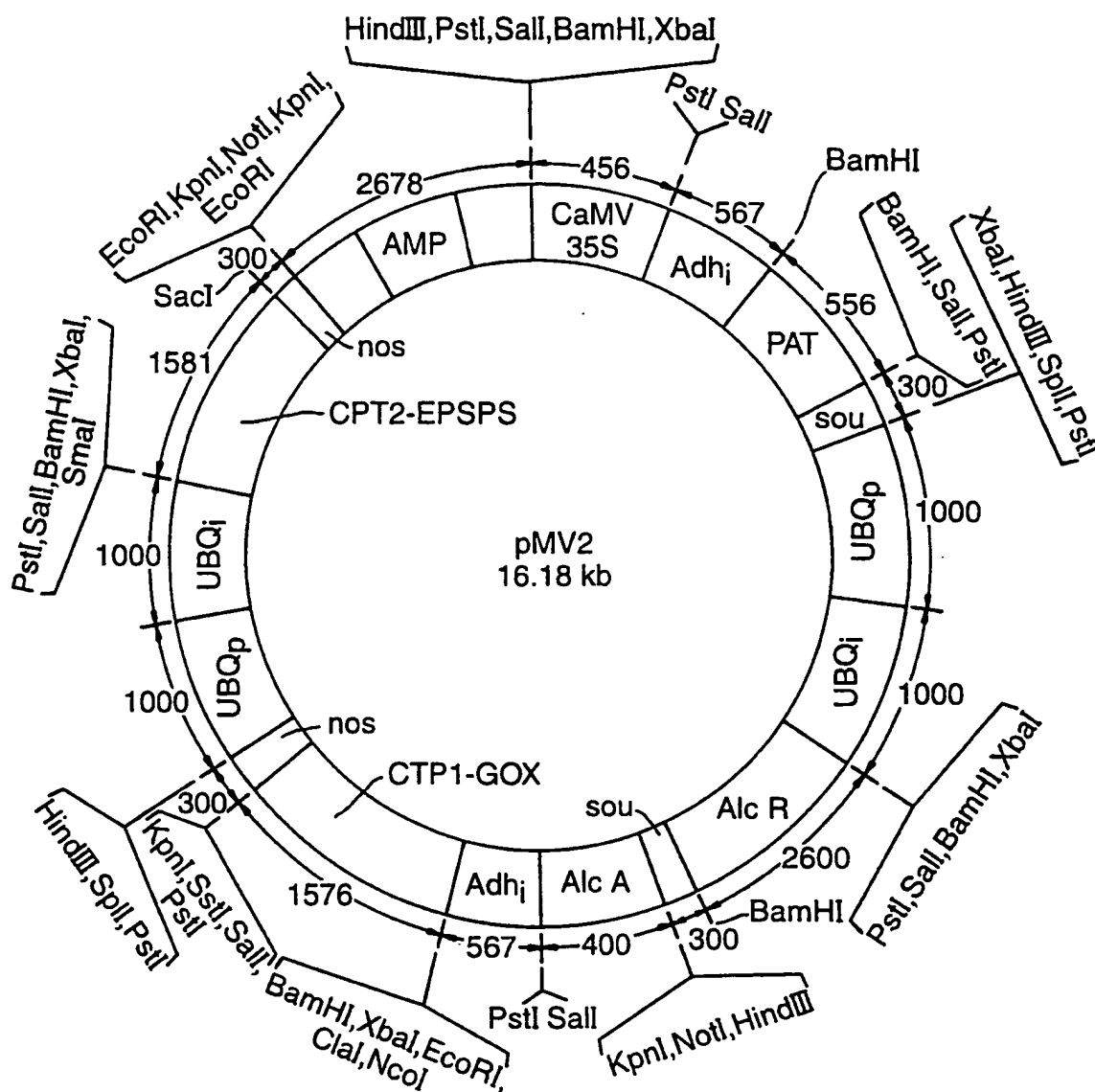
25/30

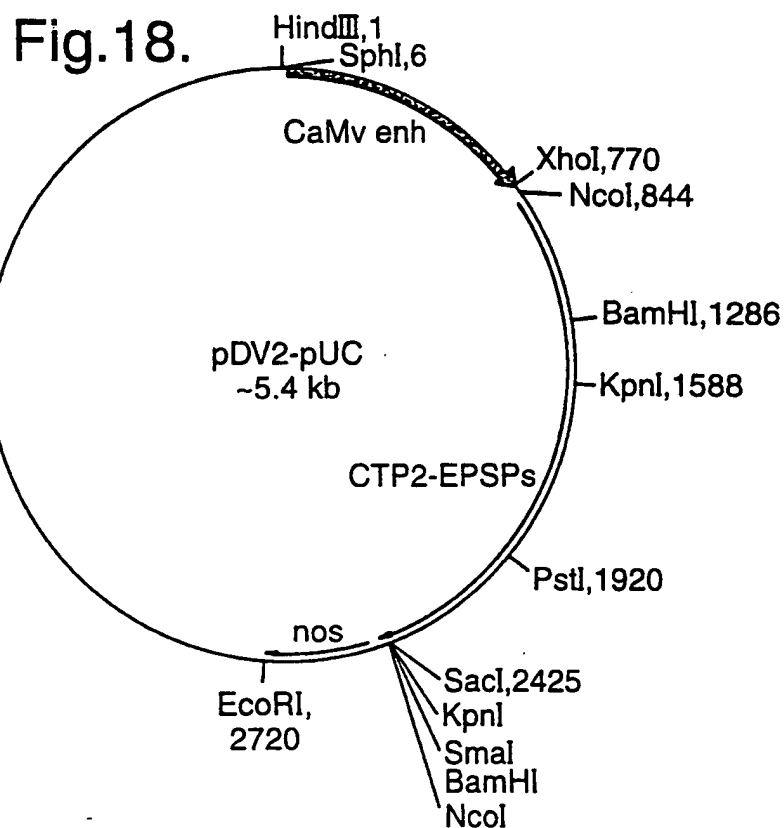
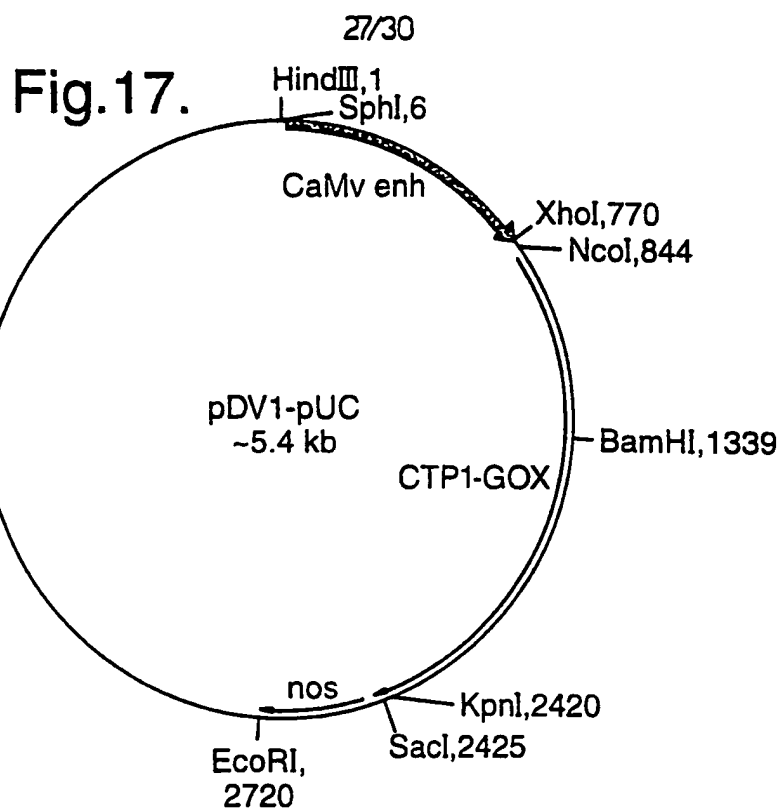
Fig.15.



26/30

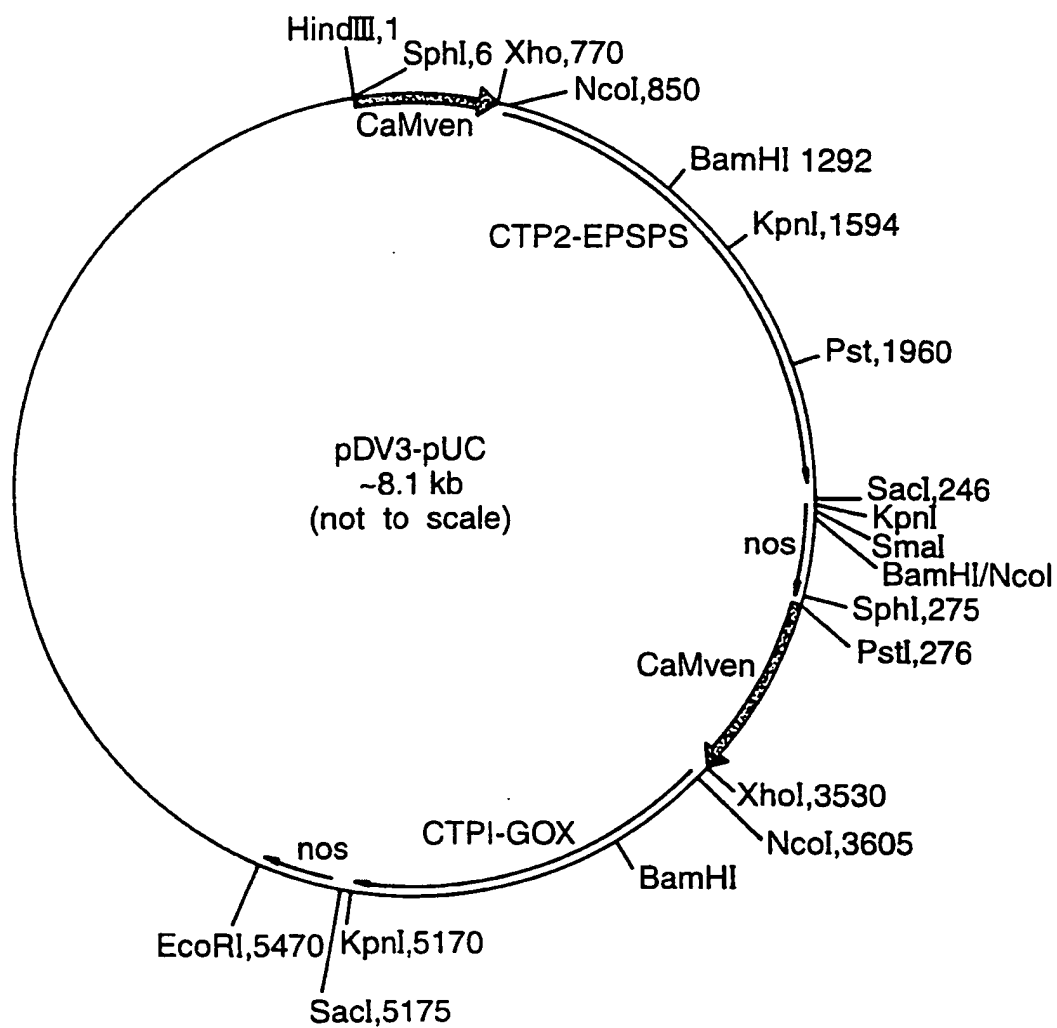
Fig.16.





28/30

Fig.19.



29/30

Fig.20.

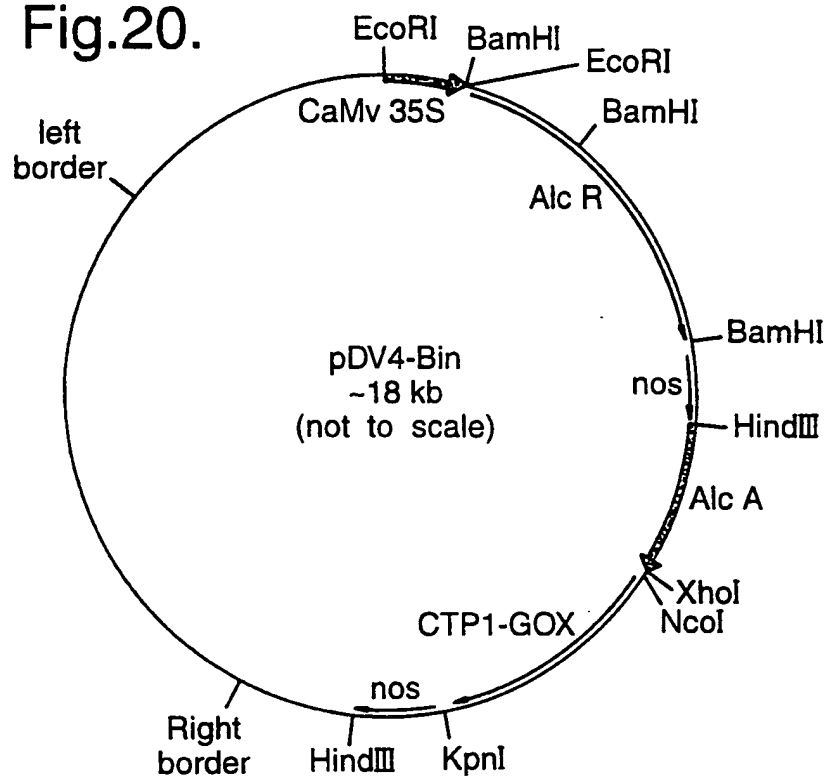
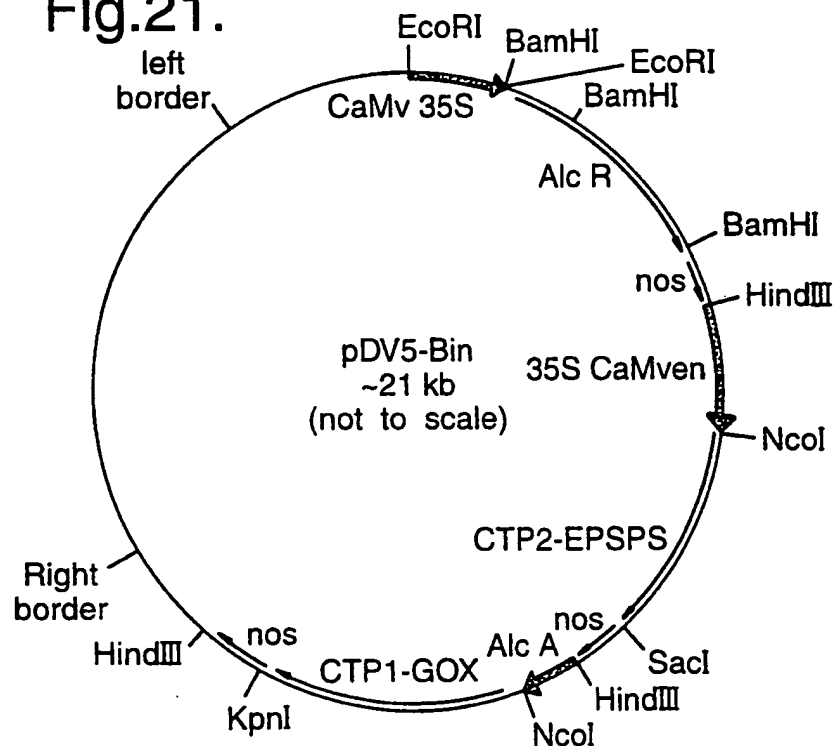
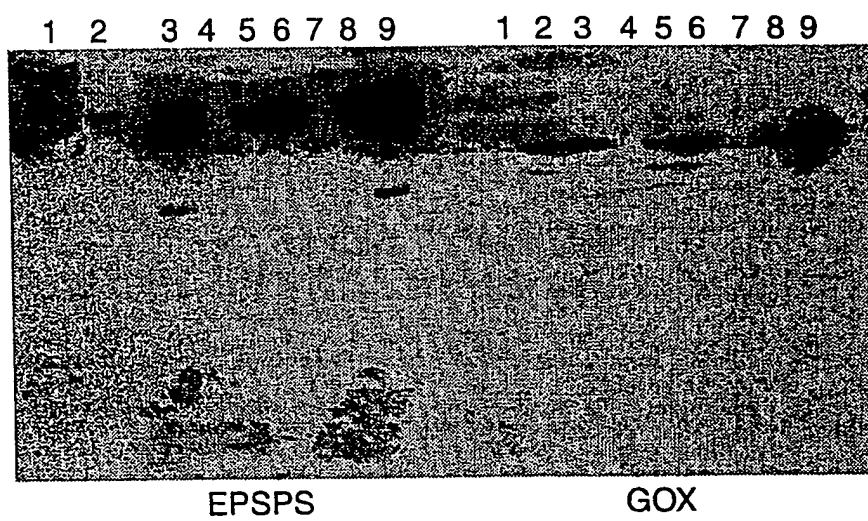


Fig.21.



30/30

Fig.22.



INTERNATIONAL SEARCH REPORT

national Application No

PCT/EP 96/01883

A. CLASSIFICATION OF SUBJECT MATTER
 IPC 6 C12N15/82 C12N5/10 A01H5/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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Y	see page 10, line 35 - line 41 see page 78, line 1 - page 82, line 40 ---	3
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X	WO,A,93 05164 (UNIV LEICESTER) 18 March 1993 see page 54, line 10 - line 18 see page 48, line 1 - line 19 --- -/--	1,3,8-12

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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Date of the actual completion of the international search

15 January 1997

Date of mailing of the international search report

29. 01. 97

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Authorized officer

Maddox, A

INTERNATIONAL SEARCH REPORT

International Application No

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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X	BIOTECHNOLOGY, vol. 5, no. 7, July 1987, pages 726-730, XP002022728 FILLATI, J.J., ET AL.: "Efficient transfer of a glyphosate tolerance gene into tomato using a binary agrobacterium tumefaciens vector" see page 729, right-hand column ---	1,2,7-14
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Information on patent family members

International Application No

PCT/88 96/01883

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